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Editorial Committee: W. T. WILLIAMS (Editor), E. ASHBY, G. E. BLACKMAN, R. BROWN, F. G. GREGORY, W. O. JAMES, W. H. PEARSALL, R. D. PRESTON, M. THOMAS.

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[*continued on p. iii of cover*

Floridean Starch

B. J. D. MEEUSE, M. ANDRIES,[1] AND J. A. WOOD[2]

(*Botany Department, University of Washington, Seattle, U.S.A.*)

Received 22 September 1959

SUMMARY

A survey was made of about 30 species of red algae from the Pacific Coast to find the best starting-material for the isolation, in pure and native form, of the controversial substance, floridean starch. *Constantinea subulifera* Setchell turned out to be the ideal alga for this purpose. The isolated starches were subjected to a number of physical, chemical, and enzymatic tests in order to bring out possible differences from other starch-family substances, such as amylopectin and glycogen, isolated from higher plants. According to all criteria applied there is no real difference between the various compounds, except for the fact that floridean starch gelatinizes only after very long boiling in water. End-group determinations with the aid of periodate show that the floridean starch molecule is a strongly branched structure somewhat comparable to glycogen.

INTRODUCTION

WITH the exception of Kylin (1913, 1943), who isolated floridean starch in native form from *Furcellaria fastigiata*, all the authors interested in this controversial substance (Colin and Augier, 1933; Colin, 1934; Barry, Halsall, and Hirst, 1949; O'Colla, 1953; Fleming, Hirst, and Manners, 1956) have contented themselves with contaminated preparations derived from aqueous solutions. The literature-data, mostly on floridean starch from *Dilsea edulis*, are thus open to some doubt, a fact which is especially serious in the field of optical rotation where conclusions as to the linkage-type of the polysaccharide might be drawn from the values recorded; it is hard to interpret the differences in the available data (Barry, Halsall, and Hirst, $+156°$; O'Colla, $+166°$; Fleming, Hirst, and Manners, $+176°$). Clearly, at this moment, more than a century after the discovery of floridean starch by Kützing (1843), a renewed and critical study of this substance, with emphasis on biological and mechanical isolation-methods, has relevance. The present report covers an attempt at such a critical study, begun by the senior author in 1953 and involving about thirty species of red algae. Two brief notes on the first results have already appeared (Meeuse and Kreger, 1954 and 1959).

MATERIAL AND METHODS

1. *Isolation methods*

In the case of *Constantinea subulifera* and *Rhodymenia pertusa*, both excellent producers of floridean starch, very pure samples of this substance can be

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obtained through methods of fractionated centrifugation, applied to the slurries obtained by grinding the fresh algae in a strong IKI-solution. Diatoms are the contaminants that are hardest to remove.

In the other cases pretreatment of the first crude starch-suspensions with *n*-butanol in a separating funnel was necessary to make the centrifugation-procedures successful. Pigments and proteins tend to accumulate in the upper (butanol) layer, whereas many of the floridean starch granules sink down to form a deposit at the base of the aqueous layer. Staining with iodine is not essential for the success of the method; before being applied to floridean starch, it has given good results in the isolation of paramylum from *Euglena* which had not been so treated (Kreger and Meeuse, 1952).

In some cases where only small amounts of material were available, and where contamination with mucilaginous substances was strong, considerable purification was achieved by using the 'robot' referred to by Meeuse and Kreger (1959). This was followed by butanol-treatment and fractionated centrifugation, as above.

2. *Purification*

From the wet, reddish-purple samples of iodine-treated starch obtained according to the above-mentioned procedures, purified floridean starch was obtained by a treatment with butanol and hot water, as follows. To eliminate the iodine, the sample (dispersed in a small volume of cold water) was shaken three times in succession with a double volume of *n*-butanol; each time the starch was reisolated by centrifugation. To eliminate diatoms and proteinaceous contaminants, the wet samples were thereupon heated for 30 minutes on a steam-bath with an equal volume of initially boiling water. This heating-period was sufficient to cause some slight swelling of the granules but was far from adequate to result in their complete gelatinization. After centrifugation (which in the case of *Constantinea subulifera* was continued for 30 minutes at 2,000 × g), 3 layers were obtained: a scummy top layer, containing some protein, a milky middle layer of suspended, slightly swollen starch-granules, and a sediment consisting of diatoms mixed with slightly gelatinized starch. The milky middle layer was collected by carefully siphoning it off, its volume was reduced by evaporation *in vacuo*, and the starch-granules were precipitated from it by adding ethanol to give a final concentration of 80 per cent. The precipitate was collected on a filter, washed with absolute ethanol followed by peroxide-free ether, and dried *in vacuo*.

3. *Suitability of various red algae for the isolation of floridean starch*

Table I indicates the usefulness of some red algae from Western Washington as floridean starch donors, on the somewhat arbitrary basis of the isolation- and purification-methods just outlined. In summary *Constantinea subulifera* is found to be excellent, *Rhodymenia pertusa* very good, *Plocamium* and the *Odonthaliae* good. All these algae are common in the area where the work was carried out. The *Ahnfeltiae*, *Laurencia spectabilis*, and *Ptilota pectinata*, al-

TABLE I

Suitability of various red algae for the isolation of floridean starch

−: not suitable. ±: doubtful. +: suitable.

Name of alga	Suitability	Remarks
Agardhiella coulteri (Harvey) Setchell	−	
Ahnfeltia concinna J. Agardh	+	
Ahnfeltia plicata (Hudson) Fries	+	
Bangia fuscopurpurea (Dillwyn) Lyngbye	−	no floridean starch
Callophyllis edentata Kylin	±	
Callophyllis flabellulata Harvey	±	
Callophyllis heanophylla Setchell	±	
Constantinea subulifera Setchell	+++	ideal species
Cryptopleura ruprechtiana (J. Agardh) Kylin	±	granules very small
Erythrophyllum delesserioides J. Agardh	±	
Farlowia mollis (Harvey and Bailey) Farlow and Setchell	±	
Gigartina spp.	−	
Gracilaria verrucosa (Hudson) Papenfuss	−	
Halosaccion glandiforme (Gmelin) Ruprecht	−	
Iridaea spp.	±	granules very small
Laurencia spectabilis Postels and Ruprecht	+	
Odonthalia floccosa (Esper) Falkenberg	+	
Odonthalia kamtschatica (Ruprecht) J. Agardh	+	
Odonthalia lyallii (Harvey) J. Agardh	+	
Odonthalia washingtonensis Kylin	+	
Opuntiella californica (Farlow) Kylin	±	
Plocamium pacificum Kylin	++	
Porphyra naiadum Anderson	−	no floridean starch
Porphyra perforata J. Agardh f. *perforata*	−	" " "
Prionitis lanceolata Harvey	−	
Prionitis lyallii Harvey	−	
Ptilota pectinata (Gunner) Kjellman	+	
Rhodomela larix (Turner) C. Agardh	−	
Rhodymenia palmata f. *palmata* (L.) Greville	±	granules very small
Rhodymenia pertusa (Postels and Ruprecht) J. Agardh	++	second best species

though potentially good, were not sufficiently abundant to be very useful. Unless stated otherwise, all the following work was done with starch obtained from *Constantinea subulifera*.

4. *Purity checks on the floridean starch isolated*

(*a*) *Anthrone reaction* (Seifter *et al.*, 1950). Desiccated samples of floridean starch, oven-dried at 106° C. for 4 hours, were compared with some very pure samples of potato starch treated similarly. Table II gives the quantities of sugar found in 0·05 ml. samples of aqueous, autoclaved solutions containing 0·1 per cent. starch.

It is clear that in terms of sugar units obtained the purity of the floridean starch and that of a very pure sample of potato starch were comparable. Admittedly the information which the test gives on contaminants of a carbohydrate nature is limited. However, no galactose could be found in the acid

TABLE II

Purity check on floridean starch with the aid of anthrone method; potato starch as reference substance

	Potato starch		Floridean starch	
	μg of sugar found per 0·05 ml.		μg of sugar found per 0·05 ml.	
	Solution I	Solution II	Solution I	Solution II
Sample 1 . .	54	53	52	52
2 . .	53	53	52	53
3 . .	54	—	53	—
4 . .	53	—	52	—
Average . .	53·5	53	52·25	52·5

Sugar ('glucose') per 100 mg. starch:
Potato starch: 107·0 mg.; 106·0 mg. Floridean starch: 104·5 mg.; 105·0 mg.

hydrolysate of the floridean starch by means of filter-paper chromatography; no admixture of uronic acids could be detected in the polysaccharide by means of the naphthoresorcinol test, and no ketoses were found with the acid resorcinol reagent.

(*b*) *Complete breakdown to glucose with the aid of Cryptochiton enzymes.* In 1958 Meeuse and Fluegel have reported that the enzymes contained in the sugar gland juice of the giant chiton, *Cryptochiton stelleri*, break starch down completely to glucose, which can be assayed with notatin (Keilin and Hartree, 1948). Application of the method to floridean starch, with potato starch as the reference material, gave the results recorded in Table III.

TABLE III

Comparison of floridean starch and potato starch, in terms of glucose liberated by Cryptochiton enzyme

	Glucose found with notatin method in Warburg vessel (mg.)	Starch from which glucose in previous column was formed (mg.)	Per cent. glucose from starch	Per cent. glucose from starch, theoretical
Floridean starch	4·69; 4·78	4·55	103; 105	105
Potato starch	5·54; 5·72	5·50	101; 104	105

The difference between the experimental and the theoretical values is well within the accuracy-limits of the methods employed. Although the remarkable specificity of the notatin-method is well known, the presence of glucose as the product of hydrolysis was in addition confirmed by the preparation of phenyl-glucosazone from the digests.

(*c*) *Optical rotation.* According to Clendenning (1945) the optical rotation of a starch can be determined with great accuracy (i.e. within 0·3 per cent.) by dissolving the substance in a calcium chloride solution of spec. gr. 1·3, which is subsequently adjusted to pH 2·0–2·5 with 0·8 per cent. acetic acid.

Proteins (if any) are precipitated with a 20 per cent. solution of $SnCl_4$. In applying Clendenning's method to floridean starch, no appreciable precipitate was produced with $SnCl_4$, this indicating that no proteinaceous impurities were present. With potato and wheat starch as reference materials the results recorded in Table IV were obtained.

TABLE IV

Specific rotation of starch from potato, wheat, and Constantinea

	Potato I	Potato II	*Constantinea*	Wheat
Degrees angular rotation (*a*)	3·91	3·90	3·93	3·89
Degrees specific rotation (α)	195·5	195·0	196·5	194·5

Thus the specific rotation found for the floridean starch is practically identical with that of potato and wheat starch. From the results obtained by Clendenning and Wright (1945) the more general statement can be made that there is no difference in rotation between floridean starch and higher plant starches. In addition to arguing strongly for the purity of the material considered this signifies that the main linkages between the glucose units in the polysaccharide chains are of the same type in the two cases.

RESULTS

1. *Morphological, optical, and microcrystalline properties of native floridean starch granules*

(*a*) *Size*. Microscopic examination of 250 granules chosen randomly from a sample of isolated *Odonthalia floccosa* starch gave the following, skew, frequency distribution for size:

Granule size	Frequency
0·5 to 1μ	Absent; probably eliminated in the purification
1–2μ	14·4%
2–3μ	16·4%
3–4μ	12·4%
4–5μ	8·8%
5–6μ	8·4%
6–7μ	6·8%
7–8μ	6·0%
8–25μ	26·8%

(*b*) *Shape and microscopically visible structure* (Pl. 1, Figs. 1, 2). The following types of 'granules' can be observed in floridean starch: shallow bowls and flat disks, somewhat angular but regular, often hexagonal, showing a distinct concentric system of layers (see Pl. 1 and Fig. 2); granules shaped like an hourglass halved lengthwise; boomerang- and banana-shaped bodies; X-shaped granules. The most reasonable explanation for the occurrence of these various shapes is the assumption that the granules of floridean starch, in contrast to the starch-granules found in higher plants, are formed *on the outer surface of*

a plastid, not in its centre, and that occasionally 'back-to-back' fusion of granules occurs. Most common are the bowl-shaped, hexagonal, or almost circular granules; the 'irregular' forms seem to occur much more often in some species (*Plocamium pacificum*, *Odonthalia floccosa*, *Iridaea spp.*) than in others (*Constantinea subulifera*, *Rhodymenia pertusa*). There seems to be no connexion between size and irregularity of shape in floridean starch granules; thus very small 'X-es' were found in *Iridaea*-species, very large ones in *Plocamium pacificum*.

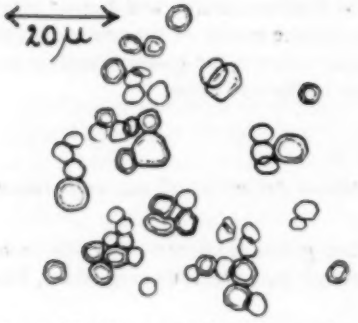

FIG. 1. Floridean starch from *Constantinea subulifera*, Setchell.

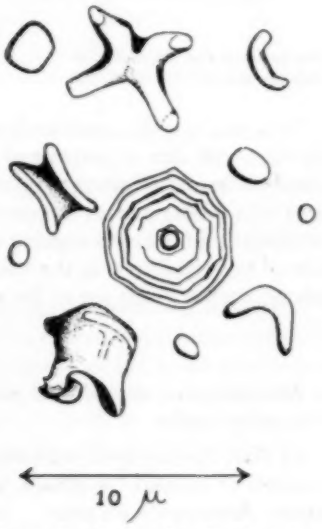

FIG. 2. Starch-granules of various shapes observed in *Plocamium pacificum*, Kylin.

(*c*) *Submicroscopic* (*microcrystalline*) *structure*. Between crossed nicol prisms granules of floridean starch show birefringence, but the phenomenon cannot always be observed easily because the bowls are so thin. In those cases where the granules are more spherical and sufficiently thick, they display the familiar dark cross, very much like, for example, potato or barley starch grains; this suggests a radial arrangement of the crystallites. The X-ray diffraction of starches from about twenty-five different red and green algae has recently been studied by Meeuse and Kreger (1959). Their results can be summarized very briefly as follows. The spectra can be grouped in four classes, which will here be designated simply as I, II, III, and IV. Class I, comprising a number of starches from green algae, corresponds with the well-known A-spectrum produced by cereal starches. *Polypodium*-starch also turned out to belong in this group. Class II corresponds with the so-called B-spectrum, displayed by 'tuber-starches' such as that from potato. The reserve materials of both red algae (*Plocamium*, *Constantinea*, *Odonthalia*) and green algae (*Chara*, *Codium*

fragile) are in this group. Class III is characterized by a ring at 4·50 A which is always conspicuous, although it may vary somewhat in definition and intensity from case to case. We shall refer to the spectra in this class as *Ulva*-type spectra. They are produced by starches from a number of green algae, by the reserve material of the moss *Mnium affine*, and by that of the red algae *Laurencia spectabilis*, *Ptilota pectinata*, and *Rhodymenia pertusa*. The spectra in class IV resemble those of the *Ulva*-type but are vaguer. They are given by starch from *Nitella* and by that of the remaining starch-producing red algae investigated. Two facts emerge clearly from this survey. (i) There are at least six floridean starches which according to their X-ray pattern are similar to, or identical with, potato starch. (ii) Those floridean starches which have a deviant pattern share this with certain reserve materials from green algae whose starch-nature has never been seriously doubted.

2. *Iodine reaction*

It is well known that the quality and the intensity of the iodine colour can, with certain reservations, be used as a clue to the chemical nature of starch-family substances.

In Fig. 3 the colours of the iodine complexes of glycogen, floridean starch from *Constantinea*, and potato starch are compared. It can be seen that the iodine complex of the algal substance is intermediate in its optical properties between the similar complex produced by the strongly branched compound, glycogen, and that formed by starch. In addition floridean starch from *Odonthalia* has been subjected to the procedure designed by McCready and Hassid (1943) for the determination of the amylose/amylopectin ratio. The percentage of the unbranched (or very slightly branched) compound, amylose, was negligible. Efforts to precipitate amylose from solutions of floridean starch with the aid of thymol (Haworth, Peat, and Sagrott, 1946) or butanol (Schoch, 1942) were unsuccessful.

3. *Differential thermal analysis*

According to Morita and Rice (1955) this method of characterizing certain organic substances 'consists in heating the substance to be studied at a constant rate with calcined alumina. The ensuing exothermic and endothermic pyrolysis reactions are electronically measured against a heat-stable reference material, so that the temperature differences between the sample and the reference material are recorded as a function of temperature.'

In 1955 the senior author, upon Dr. Morita's request, provided the latter with a sample of floridean starch from *Constantinea* for thermal analysis. The result (published by Morita in 1956) shows clearly that the properties of the algal polysaccharide are similar to those of higher plant starch. The prominent feature of the thermal curve is the endotherm at 240° C. where transglucosidation reactions occur.

4. *Breakdown by amylases*

Figs. 4 and 5 represent the action on floridean starch of salivary amylase and of a purified β-amylase; potato starch was used as a reference material.

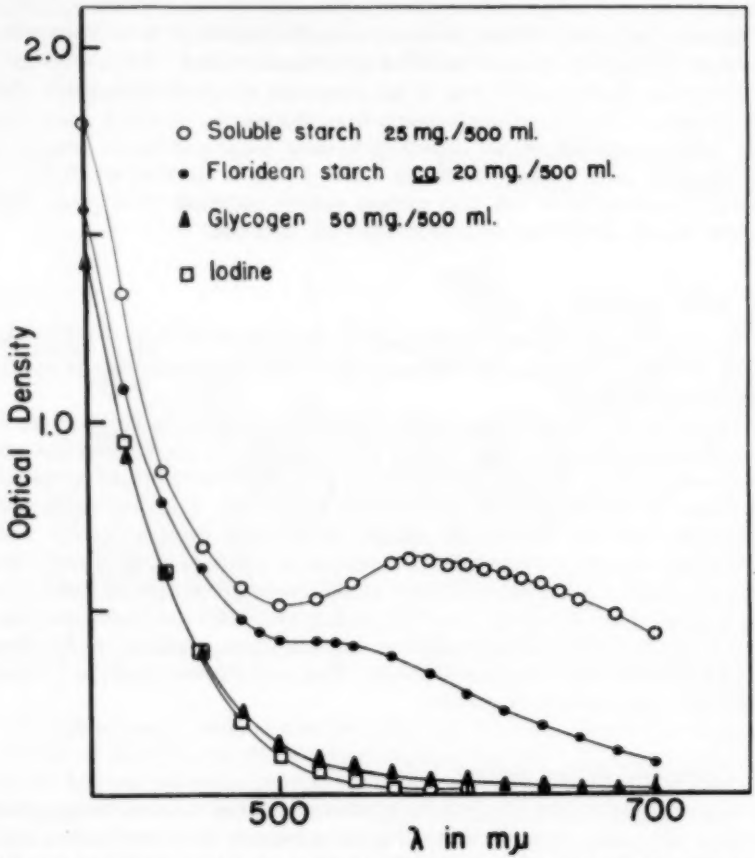

FIG. 3. Iodine colour produced by floridean starch, glycogen, and soluble starch from higher plants.

The difference between the curves for the two substrates is more pronounced in the case of β-amylase, indicating a higher degree of branching in the floridean starch than in the potato starch.

5. *Breakdown by phosphorylase*

Potato phosphorylase was fractionated with ammonium sulphate and ammonium sulphate plus NaCl, according to Fischer and Hilpert (1953); the

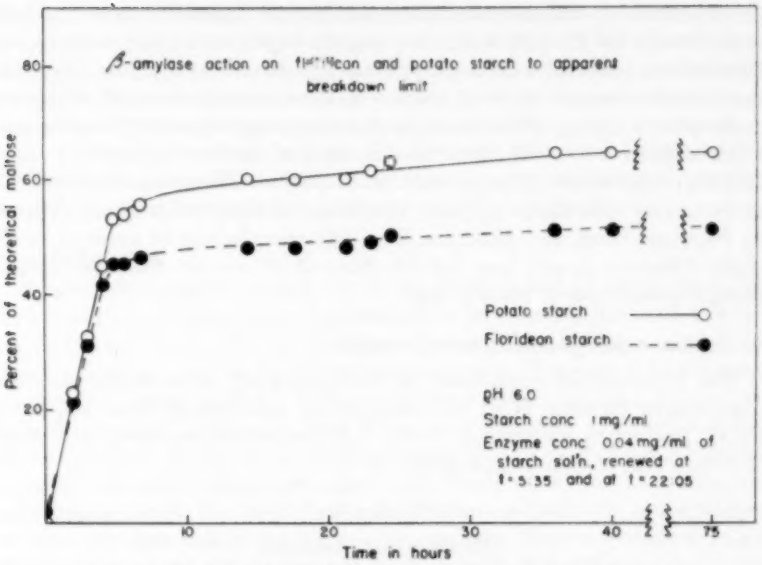
β-amylase action on floridean and potato starch to apparent breakdown limit
Percent of theoretical maltose
Time in hours
Potato starch
Floridean starch
pH 6.0
Starch conc. 1 mg./ml.
Enzyme conc. 0.04 mg./ml. of starch soln., renewed at t = 5.35 and at t = 22.05

FIG. 4. Action of alpha amylase on floridean starch and potato starch.

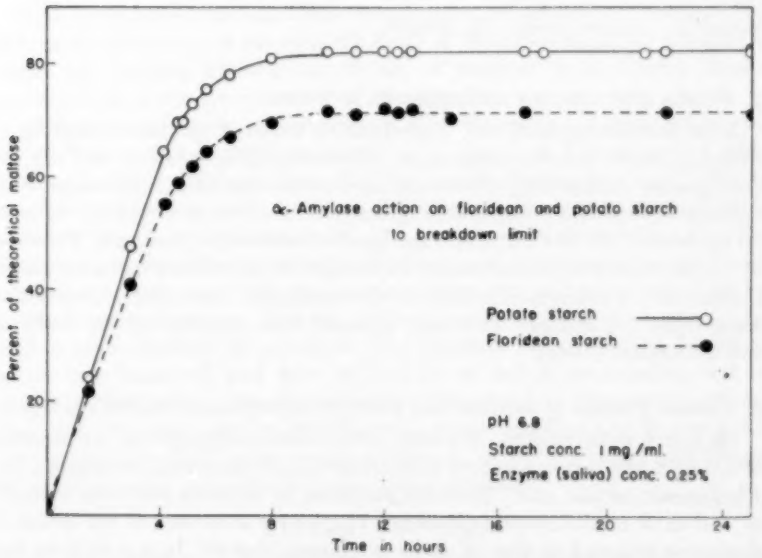

FIG. 5. Action of beta amylase on floridean starch and potato starch.

preparation of 35-fold increased purity (activity/N. basis of crude extract) was used. Except for the pH, which was slightly higher in our experiments, the digests were identical with those of Fleming, Hirst, and Manners (1956), so that in a total volume of 10 ml. there were present 2 ml. of 0·5 M. phosphate buffer pH 7·0, 25 mg. of floridean starch, and 100 mg. of potato phosphorylase. 2-ml. aliquots were withdrawn for the assay of glucose-1-phosphate. After 22 hours a conversion of 34 per cent. was recorded. This compares somewhat unfavourably with the 41 per cent. conversion of waxy maize starch obtained by Fleming, Hirst, and Manners. The difference cannot be ascribed to the slight difference in pH (see, for the effect of pH on the starch/phosphate/phosphorylase system, Hanes, 1940).

6. *Oxidation with potassium metaperiodate*

The experimental conditions for this oxidation were similar to those described by Fleming, Hirst, and Manners (cf. also Halsall, Hirst, and Jones, 1947). The results are given in Table V; the degree of branching is comparable with that of various glycogens.

TABLE V

Metaperiodate oxidation

Floridean starch from:	Weight floridean starch (mg.)	Yield of formic acid (mg.)	Glucose residues per end group
Constantinea subulifera	94·5	2·25	12
Odonthalia washingtonensis I . . .	54·0	1·05	14
Odonthalia washingtonensis II (treated with Ca-chloride) . . .	72·8	2·0	10

7. *Residue after complete oxidation with periodate*

After purification and acid hydrolysis of oxidized samples according to Fleming, Hirst, and Manning, paper chromatography revealed only 1–2 per cent. glucose (maximum). Under identical conditions soluble laminarin from *Laminaria* yielded a considerable quantity of glucose, as could be expected on the basis of the fact that it contains an abundance of 1:3 linkages. The value of 1–2 per cent. for floridean starch leaves open the possibility that a very small amount of 1:3 linkages is present in this substance. It is worthy of note that some alpha 1:3 linkages have been reported from amylopectin by Wolfrom and Thompson (1955).

8. *Possible presence of amylases and starch-phosphorylase in marine red algae*

On starch-gelatin plates (Wijsman, 1889) tests were made for the presence of α- and β-amylase in powders from freeze-dried *Odonthalia washingtonensis*, *Rhodymenia pertusa*, and *Plocamium pacificum*. In addition solutions of starch as well as of erythrodextrin (Hoskam, 1947) were subjected to the action of these powders and to that of aqueous extracts thereof. It is a striking fact indeed that the evidence for amylases that was obtained was negative or at

best extremely doubtful. One of the present authors (M. Andries) has obtained some indication of the presence of starch-phosphorylase; if confirmed, this would strengthen the arguments here presented for the lack of any fundamental difference between floridean starch and other starches. However, he also noticed some discrepancies when various red algae were compared. In view of this the present authors are of the opinion that much further work is needed; in the synthesis of starch in red algae it is very tempting to speculate on the possible role of systems involving uridine phosphates.

9. *Gelatinization*

Starches from higher plants can to a certain extent be characterized by their lower temperature-limit of gelatinization (see for this Katz and Hanson, 1934). It is well known that, in the higher plants at least, *Maranta*-starch occupies the highest position on the temperature-scale. However, it is surpassed by floridean starch; as Kylin had already observed in 1913 and 1943, a prolonged period of boiling (at least 6 hours) is required to effect complete gelatinization of the floridean starch granules. The present authors' observations on the latter's behaviour agree with those of Kylin. They consider gelatinization to be the most clear-cut difference available for distinguishing between floridean starch and higher-plant starches. However, since floridean starch represents merely the extreme in a long series, this difference can hardly be considered fundamental.

ACKNOWLEDGEMENTS

The work here reported forms an extension of paramylum studies supported by Initiative 171 funds of the State of Washington. It is a pleasure to thank Dr. Richard Fleming for the use of facilities at the Friday Harbor Laboratories of the University of Washington.

Addendum. After the completion of this manuscript our attention was drawn to an article on floridean starch by S. Peat, J. R. Turvey, and J. Môn Evans ('Isolation of nigerose from floridean starch', *Nature*, **179** (1957), 261–2), in which the claim is made that a small proportion of α-1:3 linkages exists in floridean starch, in addition to the linkages expected of amylopectin. The authors point out that, according to Wolfrom and Thompson (quoted in our present article on page 138), the same situation obtains in waxy maize amylopectin. Again, then, no clear-cut difference between floridean starch and higher plant starches is indicated. The floridean starch studied by Peat's group was 'extracted' and then purified by an iodine-precipitation and by use of cetyltrimethylammoniumbromide ('Cetavlon') to eliminate a galactan impurity. The blue value of this floridean starch was found to be 0·065, the specific rotation $[\alpha]_D^{18°}$ of its aqueous solution $+187°$—a result gratifyingly close to the value presented by us in this manuscript.

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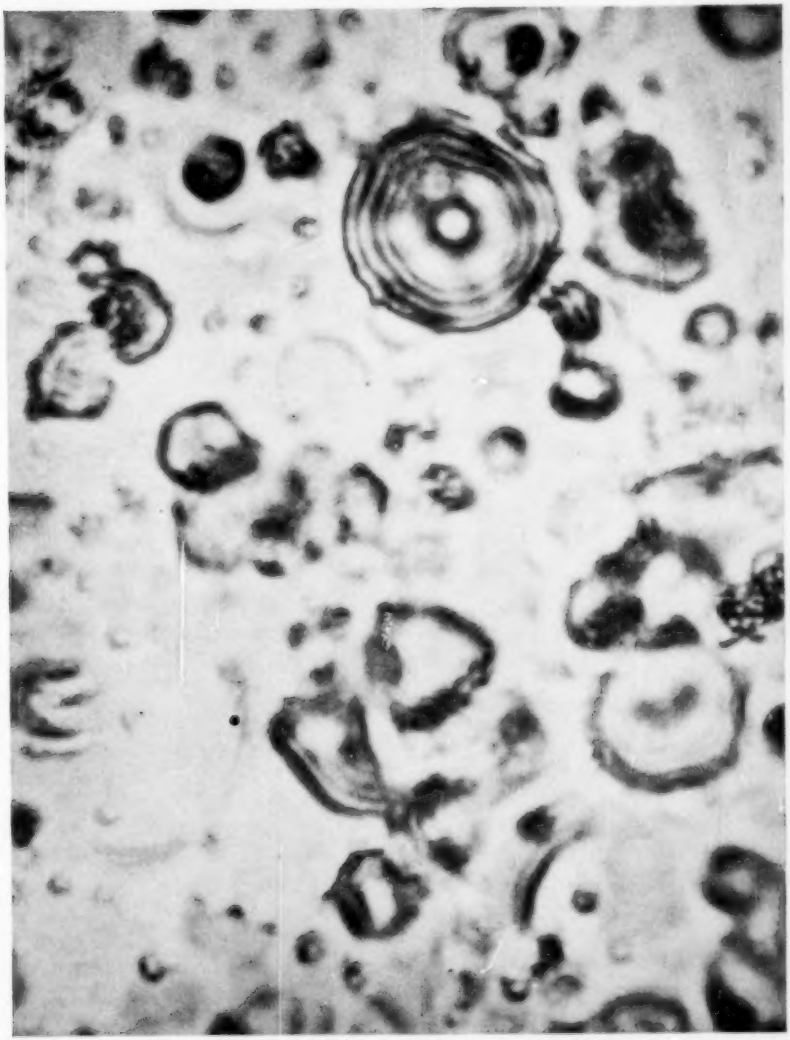

Floridean starch from *Plocamium pacificum* Kylin, showing concentric rings

B. J. D. MEEUSE, M. ANDRIES, J. A. WOOD—PLATE I

Localization and Nature of Phenolase in Sugar-beet Leaves

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SUMMARY

Phenolase activity has been found in chloroplast, mitochondrial, and soluble fractions of homogenates prepared from sugar-beet leaves. The phenolases present in the three fractions have different catecholase/cresolase ratios and respond differently to inhibitors. The chloroplasts contain about half the cresolase activity of a leaf homogenate, whereas the bulk of the catecholase activity is present in the soluble fraction.

The findings are discussed in relation to the possible mode of action of catecholase and cresolase.

INTRODUCTION

MASON (1957) suggested that the phenolase complex can be regarded as consisting of two separate activities, phenol *ortho*-hydroxylase, and *o*-diphenol dehydrogenase, which are usually termed cresolase and catecholase activities respectively.

The presence of phenolase has been shown in many different plant tissues (Mason, 1955; Bonner, 1957), usually by determining its catecholase activity. There has, however, been some disagreement as to its intracellular location in green leaf tissue. Whereas Bonner and Wildman (1946) claimed that the enzyme was in the 'soluble' fraction of spinach leaves after removal of chloroplasts, catecholase activity was stated to be located in the 'grana' in leaves of tea (Li and Bonner, 1948) and spinach beet (Arnon, 1949). Later, McClendon (1953) found catecholase activity in chloroplast, mitochondrial, and supernatant fractions from tobacco leaf, and that the concentration of enzyme was roughly proportional to the protein concentration in each fraction.

In order to clarify this apparent confusion, we have investigated the location of both catecholase and cresolase activity in fractions prepared from sugar-beet leaves. Our results show that both catecholase and cresolase activity is present in chloroplast, mitochondrial, and supernatant fractions, and that the ratio of catecholase/cresolase activity is different in all three.

MATERIALS AND METHODS

Leaves were obtained from sugar-beet plants (*Beta vulgaris* var. Sharps Klein Wanzleben E) grown in a greenhouse. The detached leaves were washed

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and kept in polythene bags at +1°. Phenolase activity of such leaves did not appear to change appreciably during storage for 3–5 weeks.

Preparation of homogenates, &c. All the operations described were carried out at −3° C. Leaf blades were detached from the midrib and macerated in a medium consisting of 0·4 M. sucrose, 0·05 M. pH 7·8 Tris, and 0·01 M. KCl (2 ml./g. leaf blade) in an M.S.E. Atomix homogenizer run at half-speed for 90 seconds. The resulting macerate was passed through two layers of cheese cloth, and centrifuged for 1 minute at 200×g to remove whole cells and debris. The supernatant fraction from this centrifugation, designated the homogenate (*H*), was centrifuged for 10 minutes at 1,200×g. The sediment (*A*) consisted mainly of chloroplasts, and the supernatant (*B*) contained fragmented chloroplasts, mitochondria, and the soluble fraction.

The chloroplast fraction (*A*) was washed by resuspending in the medium, using a glass rod with a rubber sleeve, and centrifuged for 10 minutes at 1,000×g. The final chloroplast sediment was suspended in a medium consisting of 0·5 M. sucrose, 0·067 M phosphate buffer pH 6·3, and 0·01 M. KCl. It was then centrifuged for 1 minute at 200×g to remove clumped chloroplasts.

The supernatant fraction (*B*), containing the mitochondria, was centrifuged for 30 min. at 20,000×g. The unsedimented portion (*D*) from this fractionation was used as the 'soluble' fraction, and the pellet (*C*), which contained both mitochondria and chloroplast fragments, was resuspended in the sucrose-phosphate medium by gentle stirring in a glass homogenizer. The suspension was filtered through a double layer of cheese cloth to remove clumps of material which could not be resuspended.

The supernatant (*D*) was further fractionated by centrifugation at 85,000×g in a Spinco Model L centrifuge giving a supernatant (*E*) free from microsomes and other particles.

Phenolase activity was determined by measuring oxygen uptake at 30° using conventional Warburg techniques. The flasks contained the following: in the main compartment, 1 ml. of each fraction (0·5–1·5 mg. protein, see below) in sucrose-phosphate buffer pH 6·3, and 0·5 ml. of water, or aqueous solution of inhibitor; 0·5 ml. of aqueous substrate solution in the side arm; and 0·2 ml. 20 per cent. KOH in the centre well, except where otherwise indicated. The inhibitors which were used were diethyldithiocarbamate (dieca), potassium ethylxanthate, potassium cyanide, and thiourea, all at a final concentration of 10^{-3} M. Rates were calculated after the reaction had attained maximal velocity.

Chlorophyll was estimated by the method of Arnon (1949). Proteins were determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) and nitrogen values calculated as protein/6·25.

Phenols were identified by co-chromatography with known compounds in toluene-acetic acid-water (Bate-Smith, 1954), and by their characteristic colour reactions (Swain, 1953).

All chemicals used were of the highest purity commercially available;

copper-free water, obtained by passing once-distilled water through Amberlite Monobed MB1 resin, was used throughout.

RESULTS

Activity of sub-cellular fractions. The activities of chloroplasts (*A*), mitochondria (*C*), and the supernatant (*D*) were determined towards catechol and *p*-cresol. It can be seen (Table I) that the mitochondrial fraction was the most active, and that there were marked differences between chloroplasts and mitochondria in their catecholase and cresolase activities. Not only did the catecholase/cresolase ratio in the two fractions differ, but the chloroplasts oxidized *p*-cresol with a much shorter lag phase than was shown by the mitochondria (Table II). If the ratio chlorophyll/nitrogen be taken as a criterion of purity, pure chloroplasts have high ratios and pure mitochondria low ones; Table II shows that as purer preparations of both mitochondria and chloroplasts were obtained, the differences in the catecholase/cresolase ratios of the two fractions became more pronounced.

TABLE I

Rates of oxidation of phenolic substrates by various leaf fractions. Oxygen uptake, μl./min./mg. protein of the preparation

	Catechol (5×10^{-3} M.)	*p*-Cresol (5×10^{-3} M.)	Catecholase/ cresolase ratio
Chloroplasts (*A*)	1·43	0·91	1·6
Mitochondria (*C*)	5·6	1·36	4·1
Supernatant (*D*)	2·46	0·72	3·4
Soluble fraction (*E*)	2·8	0·33	8·5

TABLE II

The effect of variations in the chlorophyll/nitrogen ratio of chloroplasts and mitochondria on their activity towards catechol and p*-cresol, shown by the catecholase/cresolase ratio and the lag phase*

	Chlorophyll/ nitrogen ratio	Catecholase/ cresolase ratio	lag phase towards *p*-cresol (min.)
Chloroplasts	1·04	1·4	5
	1·07	1·6	5
	1·11	1·06	5
	1·18	1·0	5
Mitochondria	0·83	1·8	20
	0·53	4·1	20
	0·33	4·7	20
	0·26	3·6	20

Chloroplasts and mitochondria were both disrupted by resuspending them in a hypotonic medium consisting of 0·067 M. phosphate buffer (pH 6·3) and 0·01 M. KCl. The activities of the disrupted preparations were compared with those of normal preparations; sufficient of 1·0 M. sucrose

solution was added to the flasks containing the disrupted preparations so that the final sucrose concentration was the same as in the controls. It was found (Table III) that the catecholase and cresolase activities of disrupted mitochondria decreased ~ 20 per cent., whereas those of the chloroplasts increased ~ 35 per cent. However, in both fractions the catecholase/cresolase ratio was not altered by disruption.

TABLE III

The effect of disruption on the phenolase activities of chloroplasts and mitochondria. Oxygen uptake, μl./min./mg. protein of the preparation

	Catechol (5×10^{-3} M.)	*p*-Cresol (5×10^{-3} M.)	Catecholase/cresolase
Chloroplasts			
(*a*) Whole	0·8	0·56	1·4
(*b*) Disrupted	1·04	0·78	1·3
Mitochondria			
(*a*) Whole	2·3	0·81	2·8
(*b*) Disrupted	1·9	0·72	2·6

In order to show that the activity of the enzyme towards *p*-cresol is due to *ortho*-hydroxylation, the product of the oxidation of *p*-cresol by chloroplasts, in the presence of an excess of ascorbic acid, was examined. The reaction was stopped when half the calculated amount of oxygen was consumed and the contents of the flask extracted with ether. The ether extract was examined by paper chromatography, and homocatechol (4 Methylcatechol) was shown to be present together with some unchanged *p*-cresol.

As was expected, the rate of oxidation of both catechol and cresol was dependent upon the amount of particulate fraction used (Figs. 1*a* and 1*b*). The rate of oxidation was also dependent on substrate concentration (Table IV).

TABLE IV

Rates of oxidation, by the chloroplasts and mitochondria, of different amounts of either catechol or p*-cresol. Oxygen uptake, μl./min./mg. protein of the preparation*

	Concentration of phenolic substrate		
	10^{-2} M.	5×10^{-3} M.	$2{\cdot}5 \times 10^{-3}$ M.
Chloroplasts			
Catechol	1·15	0·85	0·56
Cresol	1·28	0·80	0·32
Mitochondria			
Catechol	3·9	2·9	1·77
Cresol	1·13	0·81	0·77

Effect of ascorbic acid on phenolase activity. Since the coupled oxidation of ascorbic acid in the presence of phenols has been widely used to determine phenolase activity, the ability of chloroplasts, or mitochondria, to oxidize ascorbic acid either directly, or by a coupled oxidation with a phenolic substrate, was studied.

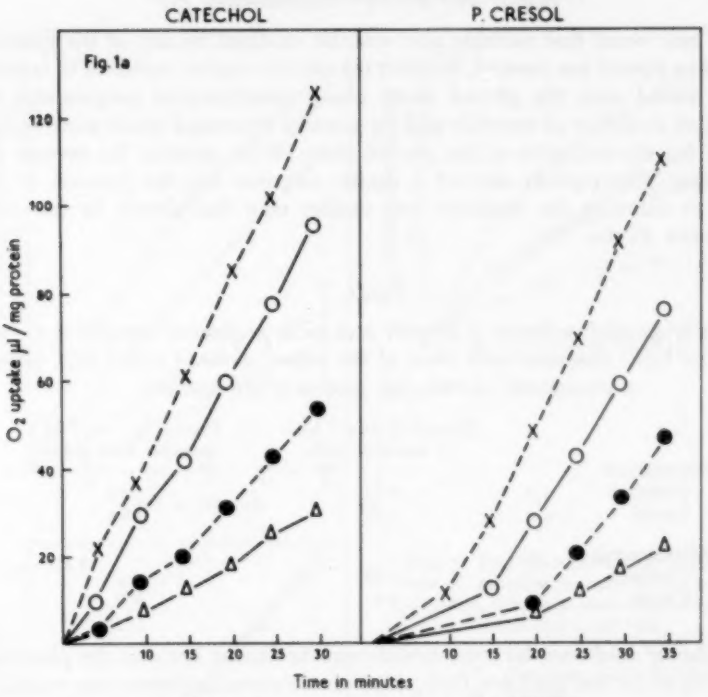


FIG. 1. The effect of enzyme concentration on the oxidation of catechol and *p*-cresol by chloroplasts and mitochondria.

1*a* Chloroplasts

×---× 1·65 mg. protein
○——○ 1·24 mg. protein
●---● 0·825 mg. protein
△——△ 0·41 mg. protein

1*b* Mitochondria

×---× 1·05 mg. protein
○——○ 0·77 mg. protein
●---● 0·525 mg. protein
△——△ 0·35 mg. protein

It was found that ascorbic acid was not oxidized by any of the fractions unless a phenol was present, and that the rate of coupled oxidation of ascorbic acid varied with the phenol used. Using mitochondrial preparations the coupled oxidation of ascorbic acid by *p*-cresol proceeded much more rapidly than did the oxidation of the phenol alone. With catechol the reverse was the case. Chloroplasts showed a similar response but the increase in rate due to coupling the oxidation was smaller than that shown by the mitochondria (Table V).

TABLE V

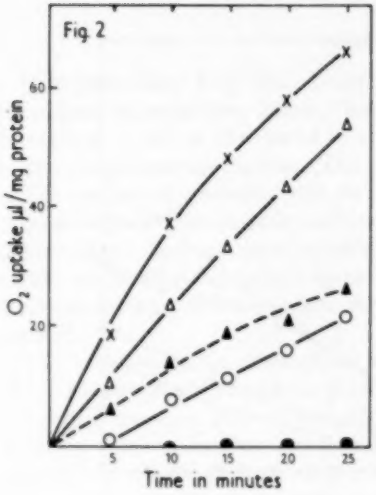
Rates of coupled oxidation of ascorbic acid in the presence of catechol or p-*cresol* (5×10^{-4} *M.*) *compared with those of the phenol alone* (5×10^{-3} *M.*). *Oxygen uptake, μl./min./mg. protein of preparation*

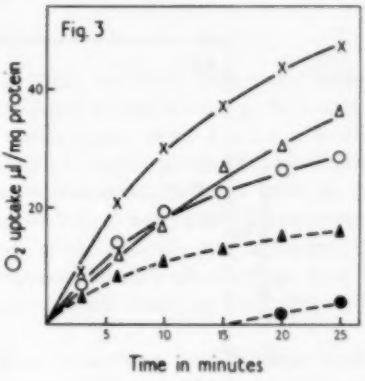
	Phenol (5×10^{-4} M.) + ascorbic acid	Phenol (5×10^{-3} M.) ascorbic acid absent
Chloroplasts		
Catechol . .	0·81	1·02
Cresol . . .	1·53	1·02
Mitochondria		
Catechol . .	1·26	3·3
Cresol . . .	2·2	0·7

Effect of inhibitors. In order to differentiate further between the phenolase activity in the leaf fractions, their response to various inhibitors was examined and the results are shown in Figs. 2–6. As can be seen the catecholase activity of all fractions responded differently to each inhibitor. The mitochondrial and supernatant catecholase showed similar responses (Figs. 2 and 3) except in the case of cyanide which was less effective in the supernatant. The chloroplast catecholase, on the other hand, was not inhibited by xanthate and only partially by dieca (Fig. 4). The inhibitors had a similar effect on cresolase in both chloroplasts and mitochondria (Figs. 5 and 6); the responses were different from those shown by the catecholase of either fraction.

Cresolase activity in chloroplasts, unlike that in the mitochondria, was totally inhibited by cyanide when an empty centre well was used. When KOH was present in the centre well, however, the cyanide inhibition was slowly reversed due to the gradual removal of cyanide from the main compartment to the centre well (Robbie, 1948), and it must be concluded that the cyanide inhibition of cresolase in chloroplasts is therefore reversible. Because cresolase activity in the soluble fraction (*D*) was so low, no inhibition experiments were carried out.

The nature of the phenolase in the supernatant fraction. In order to determine whether the phenolase in the supernatant (*D*) was genuinely soluble, and to find the contributions of the various fractions to the total phenolase activity of the original leaf homogenate, supernatants (*B*), (*D*), and (*E*) were prepared (see Methods) and their activities determined.





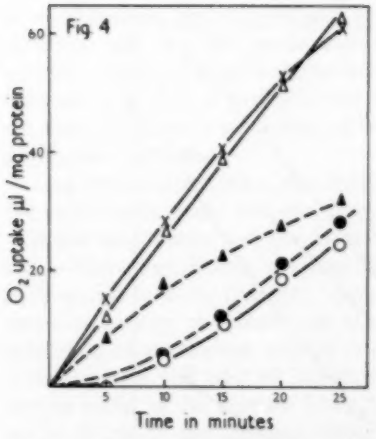


FIG. 2. The effect of inhibitors on the rate of oxidation of catechol by mitochondria. The final concentration of all inhibitors = 10^{-3} M.

×——× Control
△——△ Potassium Ethyl Xanthate
▲- - -▲ Thiourea
○——○ Potassium Cyanide
●- - -● Diecа

FIG. 3. The effect of inhibitors on the rate of oxidation of catechol by supernatant (*D*). Symbols as in Fig. 2.

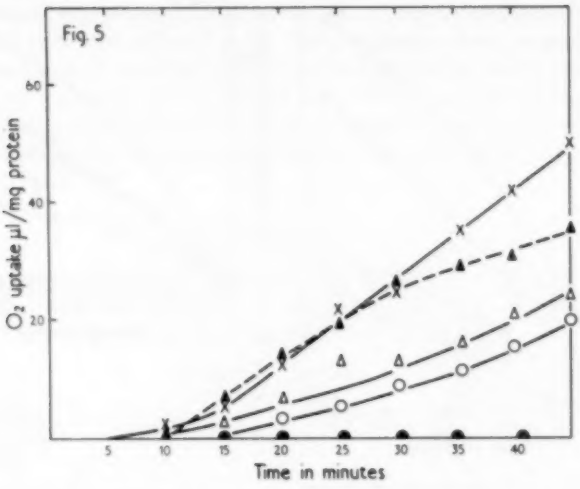
FIG. 4. The effect of inhibitors on the rate of oxidation of catechol by chloroplasts. Symbols as in Fig. 2.

It is quite clear from the results (Table VI) that the bulk of the catecholase activity is located in the soluble fraction (*E*). This is, however, not so for cresolase activity, about half of which is in the chloroplast fraction.

TABLE VI

The rate of oxidation of catechol and p-*cresol by the homogenate* (*H*), *and the reactions* (*B*), (*D*), *and* (*E*). *Phenol concentration* = 5×10^{-3} *M. Oxygen uptake, µl./min./mg. protein of preparation*

Fraction	Catechol	*p*-Cresol
H	2·67	0·6
B	2·67	0·3
D	2·67	0·35
E	3·0	0·35



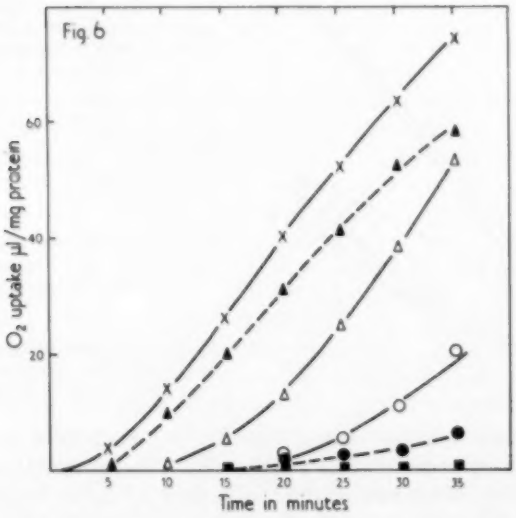


FIG. 5. The effect of inhibitors on the rate of oxidation of *p*-cresol by mitochondria. Symbols as in Fig. 2.

FIG. 6 The effect of inhibitors on the rate of oxidation of *p*-cresol by chloroplasts. Symbols as in Fig. 2 with the addition of

■- - -■ Potassium Cyanide without KOH in the centre well.

DISCUSSION

It is quite clear from the results that there are three differently located phenolases in sugar-beet leaves. One is a soluble enzyme not sedimented at 85,000 × g, a second is situated in the chloroplasts, while a third is located in the mitochondrial fraction. The earlier confusion about the localization of the enzyme is probably due to the fractionation methods used by the various authors. For example, neither Arnon (1949) nor Li and Bonner (1948) differentiated between chloroplasts and mitochondria, and although the results of McClendon (1953) agree substantially with the findings here he made no attempt to differentiate the enzymic activities for the fractions he isolated.

The enzyme in the chloroplasts differs from those in the mitochondria and in the supernatant by virtue of its low catecholase/cresolase ratio, although the actual cresolase activity of both the chloroplasts and the mitochondria is of the same order. Moreover, the two enzyme activities in each fraction respond differently to the various inhibitors.

It may, of course, be possible that there is a difference in permeability of mitochondria and chloroplasts to the different substrates and inhibitors. However, the fact that purification of chloroplasts increases their cresolase activity, while purification of mitochondria decreases such activity, strongly indicates that this is probably not the case. This is further supported by the fact that there is no change in the ratio of activities after breaking either particulate fraction.

This latter observation also indicates that the observed total activity in the supernatant could not arise from particles disrupted during preparation.

It has been shown that the oxidation product of *p*-cresol is the corresponding *o*-dihydroxyphenol, showing that the enzyme exhibits true *ortho*-hydroxylase activity (Mason, 1956, 1957). It is also interesting that the cresolase activity of chloroplasts is reversibly inhibited by cyanide, whereas inhibition of catecholase activity is not reversed under the same conditions. This supports the view of Mason (1956) that catecholase activity depends on the metal in the enzyme being in the cupric state, and cresolase activity on its being in the cuprous state. The cyanide complexes of cupric and cuprous copper evidently have different stability constants. Finally, the addition of a reducing agent, such as ascorbic acid, would reduce the metal in the enzyme to the cuprous state (Kendal, 1949), which would be expected to promote its cresolase activity (Mason, 1956), and this is borne out by the increased rate of oxidation of *p*-cresol by chloroplasts in the presence of ascorbic acid.

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ERRATA IN VOL. 10, NO. 29

On page 235, Table I: Equation (1) for Node 4 should read '$A/A_c = 0{\cdot}840 + 0{\cdot}0006A_c$'; Equation (2) for Node 4 should read '$A = 0{\cdot}89A_c$' and Equation (2) for Node 5–10 should read '$A = 0{\cdot}813A_c$'.

The Incorporation of ^{14}C into the Protein of Particles Isolated from Plant Cells

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SUMMARY

The incorporation of ^{14}C from labelled fructose, succinate, urea, and proline, by particulate preparations from dormant and tissue-cultured carrot cells, is examined. It is shown that ^{14}C is incorporated readily from proline, and less readily from fructose. No significant incorporation occurs from succinate or urea. No differences are noted between the two kinds of preparation. It is concluded that the incorporation of ^{14}C does not depend on prior transfer of the label to carbon dioxide followed by fixation of carbon dioxide, since the particles do not incorporate ^{14}C from supplied carbon dioxide.

Incorporation of ^{14}C by various fractions of dormant carrot tissue is examined, and it is established that the greatest incorporation per mg. nitrogen occurs in particles isolated at 10,000 g. A total cell homogenate fails completely to incorporate ^{14}C from proline into protein, and this may be due to suppression of the activity of the particles by a constituent of the supernatant liquid. The presence of coconut milk reduces the incorporation of ^{14}C from proline by particles sedimented at 10,000 g, and addition of a protein hydrolysate reduces it further. Hydroxyproline does not appear to compete with proline for incorporation, and in this respect the particulate preparations contrast with whole cells.

Particles from carrot tissue are shown to be more active in incorporating ^{14}C from proline than are particles extracted by the same procedure from red beet roots, potato tubers, and skunk cabbage inflorescences. They are, however, considerably less active than a mitochondrial preparation from rat liver.

It is demonstrated by paper chromatography that the bulk of the ^{14}C incorporated in the particles from carrot cells remains in proline and there is little or no conversion of proline to hydroxyproline in the preparations. The nature of the particles employed in this investigation is discussed, and their metabolism considered, in relation to the structure and activity of whole cells.

INTRODUCTION

INVESTIGATORS have recently turned to the study of isolated organelles, e.g. mitochondria and microsomes, in the attempt to localize such functions as respiration, protein synthesis, and ion uptake in particular parts of the cell.

From work first done on animal systems it has become apparent that mitochondria are the site of the main respiratory enzymes (reviewed by Hackett, 1955), whereas the principal site of protein synthesis has been identified with the particles known as microsomes (reviewed by Webster, 1955, 1957). However, much of the particulate matter isolated by centrifugation is now thought to be vesicular fragments of the endoplasmatic reticulum of the cell, and to these the minute granules of Palade adhere. Although plant mitochondria are mainly identified with the oxidative capacity of the cell, they have also been credited with the ability when isolated to incorporate ^{14}C-labelled amino-acids into protein if not actually to produce a net synthesis of protein.

Hitherto cells have been treated as though, in their physiology, they could be regarded as uniform throughout. However, a present trend is to recognize their marked heterogeneity and to emphasize that individual compartments or phases may perform distinct, but simultaneous, metabolic functions. In recent experiments using plant tissue cultures (Steward and Bidwell, 1958) cells seemed to be synthesizing protein in two distinct ways; in one of these the carbon from the sugar in the cell was incorporated more readily into protein than from the free amino-acids of the cell, whereas in the other a particular amino-acid, proline, seemed to be directly incorporated into protein. Moreover, it seemed clear from the data that particular substrates, e.g. glutamic acid, might exist in two distinct phases in the cell and participate in metabolism in different ways. One of these phases might be identified with particles, and in the present study an attempt has been made to determine the incorporation of carbon from various compounds into the protein of particles extracted from cells. Since differences have been observed between the metabolism of rapidly dividing cells and those in the dormant condition (Steward, Thompson, and Pollard, 1958) particles from both types of tissue were examined in the first instance.

The following labelled substances were used in this investigation.

1. Fructose, because the incorporation of ^{14}C from sugar into the protein of intact cells occurs.
2. Succinate, because of its importance as a respiratory intermediate.
3. Urea, because cells of cultured carrot tissue, like many plants, can utilize it as a primary source of nitrogen.
4. Proline, because this amino-acid appears to be incorporated directly and rapidly into the protein of carrot-tissue cultures.

In addition to determining the gross incorporation of ^{14}C into protein, the distribution of radioactivity among the constituent amino-acids was examined using paper chromatographic and radioautographic methods.

METHODS

Isolation of the particles. The tissue was ground in a mortar with sterile sand, and the amount of extraction medium used corresponded approximately with the amount of tissue. The extraction medium consisted of 0·03 M.

sucrose, 0·01 M. ethylene diamine tetracetate (E.D.T.A.), and 0·1 M. phosphate buffer at pH 7·0. The ground tissue was squeezed through muslin into a beaker, and the particles separated by centrifugation from the liquid which passed through the muslin. 40 grams of carrot tissue plus 30 ml. of extraction medium gave about 50 ml. of liquid for centrifugation. Broken cell residues were removed by centrifugation at a low speed (500 g) for 15 minutes. The supernatant fluid was then centrifuged at 10,000 g for 30 minutes and the precipitate so obtained, following common practice, was presumed to be composed of mitochondria. Although, for convenience, these preparations may be referred to as mitochondria, using the term in a preparative sense, they will be strictly described as the particles that separate at 10,000 g. The supernatant fluid from this centrifugation was again centrifuged at 10,000 g and the small precipitate discarded. The final supernatant fluid was then centrifuged at 50,000 g for 60 minutes, and the precipitate so obtained is referred to hereafter, again following common practice, as 'microsomes'. The 'mitochondria' were resuspended in extraction medium and reprecipitated before use. All these operations were performed at low temperature, using freezing mixtures to keep the mortar cool during the grinding procedure and a refrigerated Spinco centrifuge in the subsequent operations.

After the precipitates had been obtained, they were resuspended in a known volume of buffered sucrose by means of a glass homogenizer, and aliquots of the uniform suspension so obtained were used in the different experimental treatments. Electron photomicrographs of such preparations are shown in Plate I, and the nature of the material is discussed below (pages 162–3).

Aseptic precautions. A special attempt was made to maintain sterile conditions throughout the experiments. Tissue grown aseptically in culture was already bacteria-free, and the material from dormant tissue was obtained aseptically, using sterilized instruments in a sterile room after surface sterilizing the storage organ with hypochlorite and with all the precautions used to maintain sterility in tissue culture work. All vessels, sand, muslin, &c., used in the subsequent operations were oven-sterilized before use. Conway units, used as incubation vessels, were oven-sterilized with glass covers attached by high melting-point silicone grease. Batches of media were separately sterilized in the autoclave. All transfers of media and particulate preparations were made taking adequate precautions to ensure sterility. Bacterial counts upon the various preparations before and after incubation showed that infection or bacterial growth was negligible, except in the case of one experiment which involved preparations from skunk cabbage (see below).

Treatment of the particles. The particulate preparations were exposed to the ^{14}C-labelled compounds in the centre wells of Conway units. Alkali was placed in the outer compartment to absorb any carbon dioxide which was evolved during the experiment. Groups of Conway units were gently and continuously agitated during the experimental period of 1 or 3 hours. Generally 1 ml. aliquots of the particulate preparations were added to 1 ml. of the medium which contained the radioactive compound under test. Also present in the

incubation medium were: sucrose (0·3 M.), Mg^{++} (0·001 M. $MgSO_4$), adenosine triphosphate (0·001 M.), and sodium succinate (0·025 M.). In some experiments coconut milk at a concentration of 0·1 per cent. by volume was also added.

Analytical procedures. The material at the end of each experiment was treated in the following way: The experimental medium, with its suspended particles, was transferred to enough ethanol to make the mixture 70 per cent. ethanol by volume; this was then centrifuged. Prior analysis had shown that the particles under investigation contained very little, indeed surprisingly little, alcohol-soluble nitrogenous material. Therefore, the soluble fraction was discarded, and attention was confined to the alcohol-insoluble fraction, which is here designated protein. After centrifugation, the precipitates were washed several times by resuspension in 70 per cent. alcohol and recentrifugation. The precipitates obtained were then treated with 6 N. HCl in the autoclave for several hours. After hydrolysis of the total protein, any residual solid matter was removed by filtration and the filtrate evaporated to dryness to remove HCl. The residue was then redissolved in a suitable volume of water to produce a solution of a suitable concentration for the chromatographic separation of the constituents on paper. An amount of material which contained 30–50 micrograms of nitrogen proved to be satisfactory for each chromatogram. For each 'mitochondrial' preparation examined, enough material was available for a minimum of two chromatograms, but in the case of 'microsomes' reliance had to be placed on a single chromatogram in each experiment with certain samples. The amount of radioactivity in the sample being chromatographed was initially determined at the origin. When the amount of material permitted this, each sample was chromatographed using phenol-collidine-lutidine as solvents for one chromatogram and phenol-butanol-acetic acid for the second. The quantitative amino-acid determinations based on paper chromatography were made, using the method described initially by Thompson and Steward (1951) and as later modified in this laboratory.

To detect the amino-acids into which ^{14}C entered during the experiments, radioautographs were made from the chromatograms by the following standardized procedures. Ansco non-screen X-ray film was used in sheets of 18 by 20 in. An X-ray film was placed on each side of each chromatogram, and removed for development after convenient periods to give two different exposures.

$^{14}CO_2$ produced by the preparations was absorbed in caustic potash in the outer compartment of the Conway unit, and at the end of the experiment it was precipitated by the addition of barium chloride. The precipitated barium carbonate was centrifuged, washed, resuspended in a small amount of distilled water, and transferred to a planchette for counting. The entire sample of barium carbonate could be spread uniformly on the planchette, and the radioactivity of this preparation was determined in the usual way using a Tracerlab autoscaler.

EXPERIMENTAL RESULTS

About 2 per cent. of the protein nitrogen of the original tissue was recovered in the 'mitochondria' isolated by the above procedures. The relative amino-acid composition of the protein is shown in Table I. In comparison with the

TABLE I

Relative amounts on a nitrogen basis of different amino-acids in the hydrolysate of the protein in particles which sediment at 10,000 g obtained from tissue cultured cells of carrot phloem

	Amino-acid N as % of total protein nitrogen
Aspartic acid	5·3
Serine+glycine	9·9
Glutamic acid	12·5
Lysine	14·1
Arginine	11·2
Alanine	12·5
Tyrosine	7·1
Valine	11·3
Leucine(s)	14·4
Proline	1·6
Phenylalanine	Trace
Threonine	Trace
Methionine	Trace

whole tissue from carrot roots (Steward, Thompson, and Pollard, 1959), mitochondrial protein seems to be relatively poor in threonine, phenylalanine, and hydroxyproline. The total amount of soluble nitrogen compounds obtained from the particles before hydrolysis was too small even for chromatographic analysis, and a calculation indicated that it accounted for less than 1 per cent. of the total nitrogen of the isolated particles. Presumably, therefore, the main storehouse of soluble nitrogen compounds in plant cells is either the vacuole or the non-particulate phase of the cytoplasm, although almost all of the soluble nitrogen compounds of the 'mitochondria' *in situ* may be lost during the process of isolation.

Incorporation of ^{14}C from various substrates into mitochondria isolated from active and dormant carrot cells

In a preliminary experiment the incorporation of ^{14}C from several substrates into mitochondria prepared from carrot-tissue cultures and dormant carrot root was examined. Four substrates were used, namely fructose, succinic acid, urea, and proline, present together in all the treatments at a concentration of 0·025 M. In each treatment, one of the substrates was labelled with ^{14}C by addition of a small quantity of generally labelled fructose, succinic acid labelled in the α carbon, ^{14}C-labelled urea, or generally labelled proline. The amount of radioactivity was adjusted to 14 microcuries per Conway vessel in the case of sugar, succinate, and urea, but in the case of proline where

the specific activity of the labelled compound available was low, the radioactivity in each vessel was set at 2·5 microcuries. Coconut milk (0·1 per cent. by volume) was added to all the incubation media in this experiment. The mitochondria were exposed to the various media for 1 and 3 hours at 20° C., and the incorporation observed is recorded in Table II.

TABLE II

Radioactivity (counts per minute) in the protein of particles sedimented at 10,000 g and in the CO_2 evolved by them; the particles were prepared from dormant and actively growing tissue and were exposed to various ^{14}C-labelled compounds for 1 and 3 hours at 20° C.

Labelled compound	Time (hrs.)	Counts per protein sample	Counts in CO_2 evolved
A. *Dormant tissue*			
Proline	1	5,176	432
	3	5,176	287
Fructose	1	64	17
	3	16	18
Succinic acid	1	0	0
	3	0	0
Urea	1	0	31
	3	0	37
B. *Active tissue*			
Proline	1	7,472	250
	3	5,072	515
Fructose	1	128	14
	3	176	20
Succinic acid	1	0	0
	3	80	4
Urea	1	0	64
	3	0	56

The ability of the mitochondrial preparations to metabolize all the substrates, with the surprising but apparent exception of succinate, was demonstrated by the evolution of radioactive carbon dioxide from the compounds supplied. The comparative inability of the preparations to utilize radioactive succinate does not necessarily mean that this substance was not metabolized, since, as supplied, it was labelled only in the α carbon, and the non-radioactive carboxyl carbon may well have been evolved as carbon dioxide. However, the fact that the carbon of succinate was not completely and readily utilized again calls the identity of the particles in question.

Despite the lower total radioactivity supplied as proline, it became evident from the first that proline was by far the most effective compound in the incorporation of carbon into protein. Furthermore, the activity was incorporated with almost equal ease into particles which were isolated from dormant carrot tissue or actively growing tissue cultures. The amount of incorporation was apparently not increased when the period of exposure was extended beyond 1 hour. Fructose was the only other substrate from which appreciable incorporation occurred, but the amount was much lower than that incorporated

from proline. The differences in incorporation of radioactivity from fructose and succinate into the particles from active and dormant tissues are probably not significant.

Although the mitochondria readily released ^{14}C-labelled carbon dioxide from urea, there was no indication that radioactivity was transferred from this compound to protein. Since radioactivity appeared in carbon dioxide in the urea experiment without protein being labelled, it seems unlikely that labelling in the proline experiment occurred from radioactive CO_2 produced in any prior way from the proline molecule. This possibility will be discussed further below. The behaviour towards urea shows another point of difference between cells and particles, for the former can use C^{14}-urea to synthesize protein.

In view of the precautions taken against bacterial contamination in these experiments, and in view of the efficient washing of the precipitated protein, as shown by the fact that all traces of radioactive urea and succinate were removed from the protein preparations, the evidence strongly indicates that actual incorporation of radioactivity into protein occurred from proline and fructose. Evidence which gives further support to this contention will also be presented below.

Incorporation of ^{14}C from proline and carbon dioxide into particulate preparations

The purpose of this experiment was to test whether the incorporation of ^{14}C from generally labelled proline was due to the prior conversion to, and subsequent utilization of, radioactive carbon dioxide. To do this, a comparison was made between the incorporation of ^{14}C from CO_2 and proline under comparable conditions. CO_2 was generated from ^{14}C-labelled barium carbonate in the outer compartment of a Conway vessel containing the mitochondrial preparation. Two radioactivity levels were used; in the lower one the activity supplied in barium carbonate was about the same as that supplied in proline, and in the other ten times this activity was used. Non-radioactive proline was included in the incubation medium when carbon dioxide incorporation was being studied and compared with CO_2 incorporation in the absence of proline. Incorporation was compared in the light and in darkness, using CO_2 at the lower concentration in the absence of added proline. In other work from this laboratory it has been shown that ^{14}C from carbon dioxide can be readily incorporated into the protein of whole carrot tissue (Steward and Pollard, 1956).

It was to be expected from previous results that the particles would incorporate a substantial amount of ^{14}C from proline and this occurred, but under none of the conditions described was there an appreciable incorporation of ^{14}C from CO_2 into the protein of the particles (Table III). The small incorporation of activity from $^{14}CO_2$ was suppressed in the presence of non-radioactive proline, and this may be attributed, at least in part, to release from proline of non-radioactive carbon dioxide, which then reduced the specific

activity of the carbon dioxide absorbed. In view of the low level of CO_2 fixation which was observed, no particular significance is attached to the slight increase in CO_2 fixed in the light over that fixed in the dark, and all the CO_2 fixed is to be regarded as dark fixation.

TABLE III

Radioactivity (counts per minute) in the protein of particles sedimented at 10,000 g after extraction from dormant carrot root and exposure to either ^{14}C-labelled proline or to ^{14}C-labelled CO_2 in the presence or absence of inactive proline

Treatment	Counts
^{14}C-labelled proline	1,156
$^{14}CO_2$ inactive proline	0
$^{14}CO_2$ (10 × concentration) inactive proline	0
$^{14}CO_2$ (in light)—no proline	42
$^{14}CO_2$ (in dark)—no proline	15

Incorporation of ^{14}C-labelled proline in different particulate preparations from dormant carrot tissue

An attempt was made to determine whether particles customarily called mitochondria differed from other particles that might be obtained in their ability to incorporate ^{14}C-labelled proline into their protein. Other investigators with plant and animal materials have emphasized the importance of microsomes as sites of protein synthesis (Webster, 1955, 1957; Hoagland, Zamecnik, and Stephenson, 1957), and more recently Palade (1958) has drawn attention to even smaller granules.

In this experiment attention was confined to preparations from tissue freshly isolated from dormant roots because, as shown above, the particles when isolated from this material were as active as those obtained from the more rapidly growing tissue. The incubation medium had the same composition as that used in the previous experiments except that the concentration of proline was reduced to 0·002 M. although the total radioactivity was maintained at 2·5 μC per Conway unit. A comparison was made between the incorporation of ^{14}C into the protein of a whole tissue homogenate, by isolated particulate fractions and by combination of various fractions (see Table IV). The preparations were made in such a way that each treatment utilized material derived from approximately the same amount of tissue. When mixed particulate preparations were used, the amount of particles conveniently designated as 'mitochondria' and 'microsomes' per Conway unit was the same as that present in the single particulate preparations.

The supernatant fluid was prepared from the tissue after separating the various particulate fractions, and it was then made up to 130 ml. with 0·2 M. sucrose. Aliquots of this solution were added to the incubation medium in those treatments in which the supernatant liquid was supplied. The results of this experiment are presented in Table IV.

TABLE IV

Radioactivity (counts per minute) of the protein from various cellular fractions of dormant carrot root after exposure to ^{14}C-labelled proline for 3 hours

Tissue fraction	Counts per sample	Counts per g. fr. wt. of original tissue	Counts per mg. N in sample
Whole homogenate	0	0	0
1st precipitate at 500 g	809	73	4,170
2nd precipitate, particles at 10,000 g	1,858	290	4,690
3rd precipitate, particles at 50,000 g	329	30	1,590
Supernatant	449	1,800	3,000
Precipitates 2+3	2,338	—	3,880
Precipitate 2+supernatant	418	—	780
Precipitate 3+supernatant	389	—	1,090

From the table it is evident that radioactivity was incorporated into the protein of all the preparations, including the protein contained in the supernatant liquid. The greatest incorporation of ^{14}C per sample or per mg. N. occurred in the particles isolated at 10,000 g, although per unit fresh weight of original tissue most of the radioactivity bound in protein appeared in the supernatant liquid. Whatever basis of expression was employed, the particles at 50,000 g, i.e. 'microsomes', proved to be decidedly less active than those designated 'mitochondria' by their behaviour in the centrifuge.

A striking result was that the total cellular homogenate failed completely to incorporate activity into protein. This observation is surprising since the homogenate contains the total material from which the particulate preparations and supernatant were derived. An explanation arises from the data obtained using mixtures of particles with and without the supernatant liquid. Whereas the mixture of the 'mitochondria' and 'microsomes' showed incorporation of ^{14}C into protein which was approximately equal to the sum of the incorporation into each type of particle separately, the combination of the supernatant and the mitochondria showed less incorporation per mg. nitrogen than that obtained with the supernatant alone. This result suggests that some property of the supernatant fluid suppresses the activity of the particles, and the same may occur when the supernatant fluid and the microsomes are mixed. The latter data, however, were not conclusive, since it is possible that in the presence of microsomes the incorporation into supernatant protein was suppressed. The inhibition of ^{14}C incorporation into protein which was observed in the presence of the supernatant liquid may thus be attributed to a possible substance or substances in that liquid, and the even more complete inhibition of incorporation in the whole homogenate may thus be due to the same materials present at a much higher concentration.

The presence of such an inhibitor in dormant tissue may well account for the different behaviour of dormant and actively growing cells with respect to the incorporation of proline. This possibility could be examined by experiments similar to that described above, using extracts from actively growing tissue.

Effects of various substances on the incorporation of radioactivity from proline into the protein of the particles

A study was also made of the effect of various substances on the incorporation of radioactivity from proline into mitochondrial protein. The particulate preparations used were the same as those employed in the experiment just described. Incorporation was tested in absence of coconut milk, in the presence of coconut milk + hydroxyproline, and in the presence of coconut milk and a mixture of amino-acids derived from the hydrolysis of carrot mitochondrial protein. The results obtained are presented in Table V.

TABLE V

Radioactivity (counts per minute) of the protein in particles sedimented at 10,000 g prepared from dormant carrot root, and after the extracted particles were exposed to ^{14}C-labelled proline alone, in the presence of coconut milk, of an hydrolysate from such particles (plus coconut milk), or of hydroxyproline (0·02 M.) (plus coconut milk), for 3 hrs. at 20° C.

Treatment	Counts per sample	Counts per mg. N in sample
Mitochondria	4,258	10,075
Mitochondria + coconut milk	1,858	4,690
Mitochondria + hydrolysate + coconut milk	658	1,660
Mitochondria + hydroxyproline + coconut milk	2,698	6,200

The incorporation of radioactivity from proline was found to be greater in the absence of coconut milk than in its presence. Whereas the addition of the protein hydrolysate together with coconut milk further reduced incorporation, the addition of hydroxyproline with coconut milk seemed to increase it, although the latter effect is not statistically significant. It is probable that these observations can be explained partly on the basis that the addition of protein hydrolysate or coconut milk reduced the specific activity of the proline supplied and hence reduced the incorporation of radioactivity. Hydroxyproline evidently does not compete with proline for incorporation in the isolated particulate system, in marked contrast to the results obtained with the interaction of these substances in the growth induction of whole carrot cells (Pollard and Steward, 1959).

The activity of mitochondria from various tissues in the incorporation of ^{14}C from proline

To determine whether the phenomena described were peculiar to mitochondria derived from carrot root (*Daucus carota*) or are of more general significance, a study of mitochondria from some other organs was made. Preparations were made from the red beet root (*Beta vulgaris*), potato tuber (*Solanum tuberosum*), and the inflorescence of skunk cabbage (*Symplocarpus foetidus*), in addition to carrot root. These materials were chosen because the first two are familiar storage organs much used in physiological investigations,

and skunk cabbage mitochondria are known to be very active metabolically. Potato and beet tissues lack the orange chromatophores which may be a complicating feature in the preparation of particles from carrot. Similar amounts of tissue were used as in the experiments with carrot particulates, and the experimental media were also similar. From the results obtained (Table VI) it

TABLE VI

Radioactivity (counts per minute) of the protein particles, sedimented at 10,000 g, which were extracted from various organs and then exposed to ^{14}C-labelled proline for 3 hrs. at 20° C.

Tissue	Counts per g. fresh wt. of tissue extracted	Counts per mg. N in sample
Carrot	290	4,690
Beet	100	2,410
Potato	71	540
Skunk cabbage	82	300
Rat liver	10,740	39,400

can be seen that the particulates from the carrot root incorporated ^{14}C the most actively, the activity being expressed either per unit of nitrogen in the preparations, or per unit weight of tissue from which the mitochondria were derived. Although the skunk cabbage inflorescence produced a rich yield of mitochondria, these were not very active in the incorporation of proline into protein.

For comparative purposes, a preparation of rat liver mitochondria was exposed to ^{14}C-labelled proline in the same manner as that described above, and the incorporation of radioactivity was determined. The data (Table VI) showed that these particles were far more active in the incorporation of proline into protein than those from any of the plant sources examined. This again suggests that the particles from plants may not be entirely homologous with those cytologically recognizable, and generally described as mitochondria.

Site of the radioactivity in the protein after incorporation from proline

Two-directional chromatograms were made on many of the protein hydrolysates obtained in the above experiments, and by subsequent radioautography the location of the radioactivity was determined. In most cases it was observed that the bulk of the radioactivity occurred in proline (Plate II A), and it was notably absent from hydroxyproline, although the intact cells convert the proline after incorporation into hydroxyproline (Plate II B).

In the first experiment performed, however, a wider range of amino-acids became rather weakly labelled. This experiment was unique in that urea, fructose, succinate, and proline were present together in the experimental media, though only one of those was labelled in any given treatment. The only feature of this experiment which has proved to be consistent is the greater incorporation of ^{14}C proline into the protein than of any of the other substrates.

In all other respects, namely the very low level of total incorporation of ^{14}C from proline and the tendency toward general distribution of this label, the experiment proved to be an atypical one, and it is now known that this is due to an effect of urea autoclaved with the medium. When urea is treated in this way, it suppresses the uptake of ^{14}C proline by the tissue. In retrospect, however, it is fortunate that after a first pilot experiment the ^{14}C substrates were added *singly* to the experimental solutions, so that the interference due to autoclaved urea upon proline absorption and utilization did not obtain. Subsequent investigation has, however, shown that ^{14}C may be transferred into many other compounds if experimental media are autoclaved in the presence of urea, and among these artifacts produced by autoclaving urea are substances which selectively inhibit the incorporation of ^{14}C proline (unpublished data of Pollard and Lyndon with one of us). In none of the experiments was any ^{14}C activity detected in hydroxyproline of the protein hydrolysates, and in this respect the cell-free preparations contrast sharply with the behaviour of intact cells (Pollard and Steward, 1959).

DISCUSSION

In any work with subcellular particles the most important question is the interpretation of the particles themselves. The fractions designated 'mitochondria' or 'microsomes' in this work were initially so described because they were isolated by the standard procedures for the preparation of these organelles for experiment.

Examination by the electron microscope revealed the form of the particles which at first were designated as mitochondria. The material first designated as microsomes did not yield pictures with sufficiently distinct characteristics. The electron microscopy of plant cytoplasm has recently made rapid strides, even since these experiments were done. Workers in this field (Muhlethaler, Whaley, Manton) all seem to agree that there are several kinds of vesicular systems in the cytoplasm—the vacuole and its precursors, the equivalent of the Golgi bodies or apparatus, and the mitochondria, but there also ramifies through the ground mass of cytoplasm a system of minute tubes or canals (the endoplasmatic reticulum) which may serve as channels for the passage of materials to and fro from the plasma membranes which border the cytoplasm.

The structure of mitochondria *in situ* is now well known, for these more or less ovoid bodies contain an elaborate system of invaginations termed 'cristae'. This structure is very prominent in mitochondria of animal cells, but may also be seen in those of plants (Whaley *et al.*, 1959; Lund *et al.*, 1958). It is true that the best cytological detail is obtained in mitochondria as they are seen in cells which are far less highly vacuolated than those of carrot parenchyma, or the tissue cultures derived from them, but it is probable that, *in situ*, similar organelles in the carrot would exhibit similar features.

Plate I shows the structure of the isolated particles as revealed by the electron microscope. The first and conspicuous point is that these small and

uniform particles lack internal structure, for they do not show the internal plate-like system of 'cristae' which appears in typical mitochondria which are critically fixed and sectioned. Despite the control of osmotic pressure and of pH by the generally accepted methods, the uniformly rounded appearance of these particles, as seen in the photograph, suggests that they have been distorted by swelling. Moreover, there adhere to the surface of these rather empty globular vesicles many minute granules, which may be compared to those now commonly designated Palade particles, that is, adhering granules of pentose nucleic acid.

Thus the electron microscopic examination of the particles as isolated which, following usual practice, could have been termed mitochondria does not confirm them as particles that actually existed as such in the intact cell. On the contrary, it seems to emphasize that they represent very drastic distortions imposed by the technique of isolation, which necessarily destroyed the elaborate vesicular system that no doubt occurred *in vivo*. In fact, the appearance of these rather empty globular vesicles, with their adhering granules, suggests that they derived from a relatively coarse reticular system of strands through the cytoplasm, which, when fragmented, 'rounded up' to spherical vesicles with adhering granules. In this sense, the homogenate is to be regarded as a more general cellular homogenate, free from large particles, but it is not in any cytological sense a system of mitochondria as they exist *in situ*.

The particulate preparations used here seem, therefore, to have been composed of spherical fragments of endoplasmatic reticulum to which copious granules adhere. One should, therefore, not be surprised if the metabolic behaviour of these particles differs from that of the cell as a whole; and indeed if, in essential respects, they had duplicated the behaviour of the whole cell, this would have seemed to be more than could reasonably have been expected.

As shown above, the subcellular particles can incorporate proline into their constituent protein, and this may well require synthesis of protein *de novo*. It is relevant here that Palade (1958) now regards the small particles which adhere to the endoplasmatic reticulum as the units of synthesis in the cell. The presence of such granules in the preparations, which utilize ^{14}C proline and build it into protein, would be consistent with this idea. Unlike the intact cells, however, these vesicular particles do not convert ^{14}C proline in the protein to hydroxyproline. If similar particles are isolated from cells which have previously absorbed ^{14}C proline and, while still intact, converted it to hydroxyproline, then they do contain some hydroxyproline in their protein; this occurs approximately in the same ratio to proline as in the whole cell.

One could visualize, therefore, that the inert hydroxyproline containing protein might be formed in the wall of the canals as they develop in the cytoplasm to form the endoplasmatic reticulum. Perhaps synthesis occurs in the adhering granules and hydroxylation takes place as the protein is 'spun' out in the form of the long tubular threads. If this were to be so, one could readily see that ths isolated spherical vesicle, with its adhering granules, might

duplicate any features due to the granules *per se*, but not any behaviour which is attributable to the reticulum, the form and function of which is destroyed by the isolation procedures.

In the case of particles isolated from carrot cells, incorporation is complete within one hour or less; this is consistent with the observations of Webster (1955) to the effect that incorporation of radioactivity into microsomes reached its maximum in a fraction of an hour.

The incorporation of ^{14}C as proline into the protein, which has been observed, does not necessarily prove that a net synthesis of protein occurred in the preparations. In brief experiments of this kind, no clear distinction can be made between the synthesis of new protein and the interchange of animo-acids in already existing protein. Incorporation has been observed when proline was the only amino-acid added to the medium. The possibility that other amino-acids originated by breakdown in the mitochondria, in quantity sufficient to be used with proline in a resynthesis of protein, exists but is not proved. Whatever the explanation of these observations, whether in terms of incorporation or new synthesis, they must nevertheless have some relevance to the synthesis of protein in the cell.

The experiments demonstrate that the particulate preparations not only incorporate proline into protein, but they also metabolize proline to carbon dioxide. In these two respects the subcellular particles possess capacities which are also present in the intact cell. The particles, however, differ from the growing cells from which they were obtained in their *inability* to transform proline to hydroxyproline. Hydroxyproline is a characteristic constituent of certain kinds of protein, e.g. collagen, which are not necessarily present in mitochondria nor even generally present in plants. Since the conversion of proline to hydroxyproline occurs in connexion with a protein moiety of cells which is metabolically inert, it is not perhaps surprising that the protein of the particles as isolated behaves differently. This difference underlines the fact that the behaviour of even similar protein may vary with its location in the cell and that the protein formed by isolated particles may differ structurally and functionally from the inert protein in the cells.

The importance of the isolated particles as representative of the sites of protein synthesis in the intact cell must still remain in doubt. Present indications by some workers are that particles called microsomes may exceed true mitochondria in the ability to synthesize protein. Present indications are that the minute Palade particles may be the ultimate site of synthesis. The present experiments are of value, however, because they demonstrate that the synthetic capacity of the isolated particles commonly called mitochondria, but here referred to as part of the cytoplasmic reticulum, is not negligible. That these particles represent a part of the cell which is at least capable of synthesizing its constituent proteins on suitable templates and with the use of metabolic energy seems evident. The experimental observations also indicate that conditions in the surrounding cytoplasm, particularly the presence of specific inhibitory substances, may profoundly modify this synthetic capacity.

It seems, however, very necessary to seek assurances on the morphological nature of the isolated particles commonly termed mitochondria before one can properly relate phenomena observed after they have been isolated to organelles as they exist *in situ* in the living cell. Moreover, the extreme brevity of the period during which physiological activity is retained in such particles seems to indicate that they have suffered great distortion. In this respect the measure of success here achieved in the incorporation of a particular amino-acid into the protein of isolated particles testifies again to their undoubted metabolic activity, as indeed does their ability to convert the carbon of certain compounds to carbon dioxide. This undoubted activity, however, should not obscure the equally obvious fact that it is as appropriate to stress the differences as the similarities between the behaviour of particles and cells. This is apparent with reference to the continued duration of the synthetic activity in the cell, the large overall amount of proline converted to protein by the cells as compared with the particles, and last, but not least, the failure of the isolated particles to convert proline to hydroxyproline in their protein.

ACKNOWLEDGEMENTS

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Technical assistance with the tissue-culture work was provided by Miss K. Mears, and with the chromatography and radioautography by Mrs. R. Greenwood. This help, which is here gratefully acknowledged, was made possible by a grant from the National Cancer Institute of the U.S. Department of Health, Education, and Welfare to one of us (F. C. S.)

The examination by the electron microscope of the particles used in this work was done by Dr. K. Muhlethaler of Zurich as part of a co-operative study of the tissue-cultured cells of carrot being done with one of us (F. C. S.). For permission to make use of this figure the authors are grateful.

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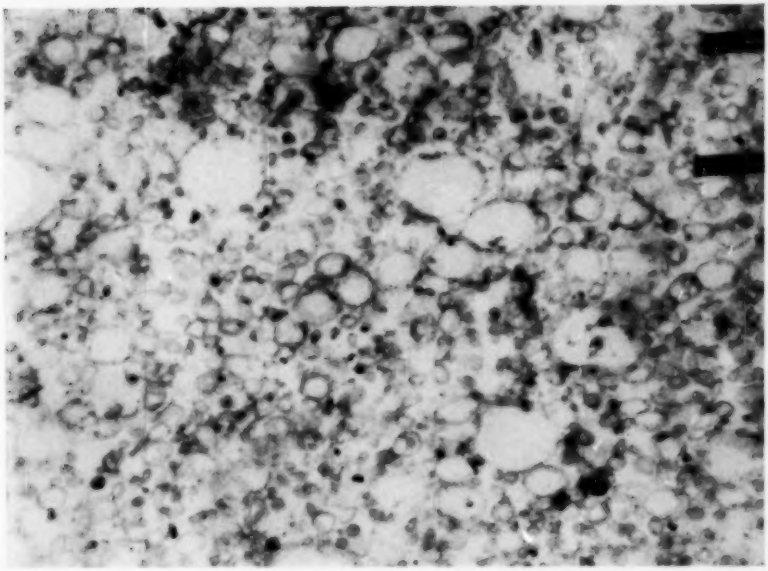

FIG. I *a*. Particles as isolated from original tissue of carrot-root phloem and sedimented at 10,000 g.: preparation contained orange-red pigment in chromoplasts. Mag. × 15,500

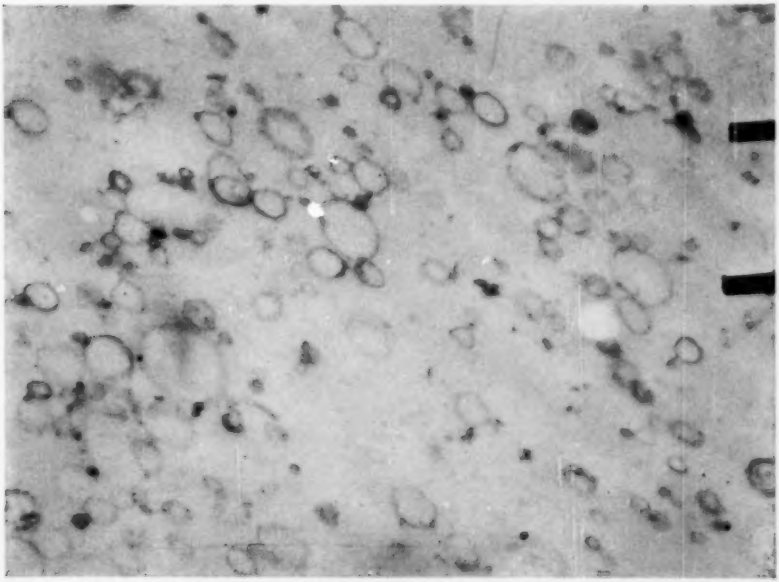

FIG. I *b*. Particles as isolated from cultured carrot tissue, sedimented at 10,000 g.: this preparation formed a pellet which was free of chromoplast pigment. Mag. × 20,000

J. F. SUTCLIFFE, E. G. BOLLARD, F. C. STEWARD—PLATE I

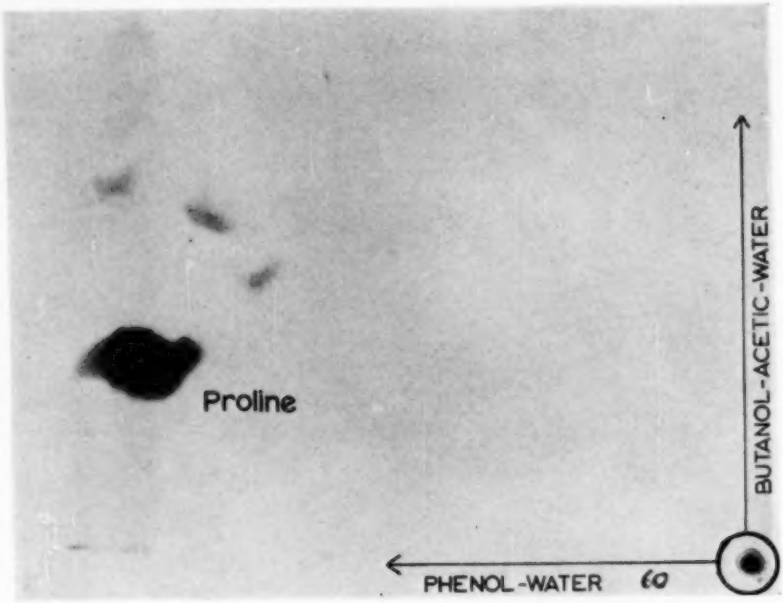

FIG. II *a*. Radioautograph of a chromatogram of a protein hydrolysate showing incorporation of ^{14}C into the protein of particles, sedimented at 10,000 g., from a homogenate of secondary phloem of carrot root. Note that the proline in the protein is not converted to hydroxyproline

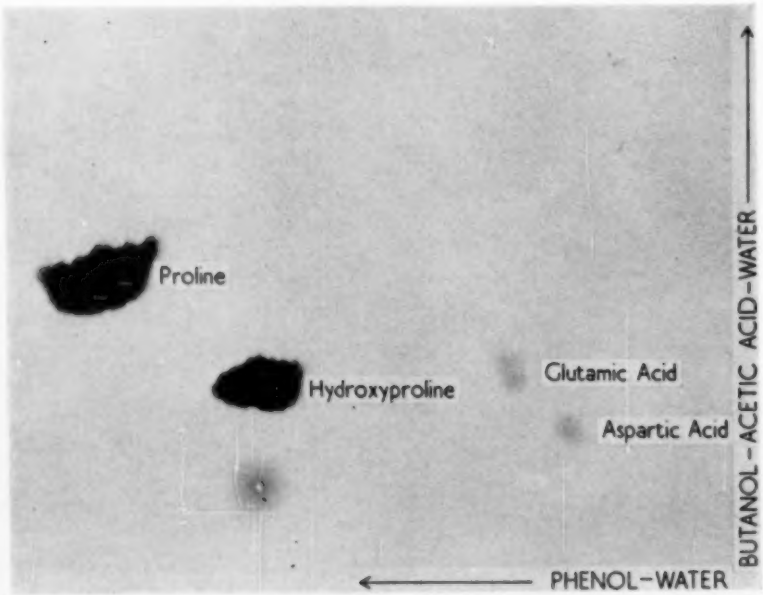

FIG. II *b*. Radioautograph of a chromatogram of a protein hydrolysate showing incorporation of ^{14}C into the protein of cells of secondary phloem of carrot root. Note that proline is converted to hydroxyproline in the protein of the intact cell. Particles sedimented at 10,000 g. after incorporation of ^{14}C had a ^{14}C-proline to ^{14}C-hydroxyproline ratio comparable to that of the protein of the whole cell

J. F. SUTCLIFFE, E. G. BOLLARD, F. C. STEWARD—PLATE II

Chelation in Auxin Action

I. A STUDY OF THE INTERACTIONS OF 3-INDOLYLACETIC ACID AND SYNTHETIC CHELATING AGENTS AS AFFECTING THE GROWTH OF WHEAT ROOTS AND COLEOPTILE SECTIONS

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SUMMARY

Factorial experiments have been carried out on the effects, upon growth of roots of intact wheat seedlings and growth of wheat coleoptile sections, of different concentrations of 3-indolylacetic acid (IAA) and various known chelating agents. These have demonstrated a similar mutual antagonism between pairs of agents whether these are IAA and a single known chelating agent or two known chelating agents. This interaction takes the form that either agent alone in 'high' concentration severely inhibits growth but this inhibitory effect is almost or entirely removed by the presence of one-millionth the concentration of the other agent; when both agents are present in 'high' concentration the inhibition is again severe. The substitution of a non-chelating analogue for one of the agents either destroys the mutual character of the antagonism or entirely prevents either agent at low concentration from reducing measurably the inhibition caused by high concentration of the other. The fact that IAA interacts with known chelating agents, in controlling the growth both of roots and coleoptile sections, in the same unexpected and symmetrical way that these interact with each other, is held strongly to support the hypothesis that it is here itself acting as a chelating or complexing agent; the absence of such interactions with a non-chelating analogue makes this the more convincing. These results are concerned with the removal of growth inhibition, due to supra-optimal concentrations of one agent, by minute proportions of another; it cannot be regarded as proven that the promotion of growth by IAA in the absence of another agent is also due to chelation or complex formation. This seems probable, however, when the findings here presented are taken in conjunction with the accumulating evidence that IAA and other auxins can form complexes or chelates with metals *in vitro*, and with the finding already published in detail that the eight chelating agents tried promoted growth in the wheat coleoptile test.

The main criticisms to which this hypothesis has been subjected have been concerned with the relative magnitudes of effects of IAA and chelating agents upon growth, with the low stability constants of metal complexes with IAA and other auxins, with the lack of parallelism between stability constants and growth-promoting activity, and with the fact that one chelating agent (ethylenediaminetetraacetic acid; EDTA) has been found inactive in certain growth tests. A series of factorial experiments comparing the authors' techniques (which are here

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[2] The experiments described form part of the work presented for the degree of Ph.D. of London University; the investigation was supported by a grant from the Agricultural Research Council.

described in detail), chemicals, and strain of wheat with those used by Fawcett *et al.* (1956) demonstrate that the discrepancies found, both as regards magnitudes of effects of IAA and EDTA and optimal concentrations, were partly due to differences in strain but mainly to differences of technique. It is considered that 'foreign' molecules such as EDTA are likely to have side effects, which may well differ in different strains or tests; competition with internal chelators (Burström and Tullin, 1957) is also likely to differ; differences in rate of penetration and steric hindrance may also be involved. For these reasons effective chelating activity *in vivo* may be very different from that *in vitro* and in the first instance the magnitudes of growth-promoting effects of chelating agents (which may indeed be the net result of stimulatory and inhibitory processes) seem less important than the fact that they are found in so many instances.

Possible ways in which IAA and other growth substances may regulate growth by chelation or complex-formation are discussed.

INTRODUCTION

ALTHOUGH the chemical identity of what is usually considered to be the principal naturally occurring plant hormone, 3-indolylacetic acid (IAA), has been known for about a quarter of a century, it cannot be said that the vast amount of investigation devoted to its effects, and to those of more or less similar synthetic molecules, has done much to elucidate the chemical mechanism by which it stimulates or inhibits plant growth. Speculation on auxin action has shown the following trends: first, the suggestion that auxin causes water uptake, and so growth, by increasing the osmotic pressure and hence turgor (Czaja, 1935); secondly, that it renders the cell wall more plastic (Heyn, 1940), i.e. reduces wall pressure; thirdly, various hypotheses that an auxin acts as an enzyme co-factor, e.g. with 2-point attachment (Foster *et al.*, 1952) or 3-point attachment (Fawcett *et al.*, 1952), and that it stimulates 'active' uptake of water (Hackett and Thimann, 1952), i.e. increases turgor; fourthly, that auxin reduces the rigidity of the pectic compounds in the cell wall (Bennet-Clark, 1956), i.e. once more that it reduces wall pressure. It would be out of place here to discuss the experimental evidence for and against these various views—suffice it to say that as far as the writers are aware no detailed suggestions have been embodied as to how the structure of the auxin molecule enables it to carry out these functions, or what, for example, is the nature of the 2- or 3-point attachments mentioned above.[1] Similarly, the set of 'rules' drawn up for the structure of an auxin molecule (first by Koepfli, Thimann, and Went, 1938), as a result of investigation of the growth-promoting activity of great numbers of synthetic compounds, gives little indication of how a molecule consisting of an unsaturated ring, separated from a carboxyl by at least one carbon and lying in a different plane from it, can produce such remarkable effects on growth.

Any clue as to what *properties* (rather than structure) of an auxin molecule are concerned in growth regulation is therefore welcome as a guide to future fields of research. Such a clue has, it is believed, been found in the discoveries

[1] The hypothesis of formation of a thiol ester with CoA (Leopold and Guernsey, 1953) should be mentioned as one in which a specific compound is postulated.

that many synthetic chelating agents can act as growth promoters in the wheat coleoptile straight growth test (Heath and Clark, 1956*a*), that IAA can chelate, or form complexes with, copper and ferric iron *in vitro* (Cohen *et al.*, 1958; Recaldin and Heath, 1958), that α-naphthyl acetic acid (Cohen *et al.*, l.c.; Armarego *et al.*, 1959) and 2:4-dichlorophenoxyacetic acid (Armarego *et al.*, l.c.) can do likewise as far as copper is concerned, and that in controlling the growth of both coleoptile sections and intact roots IAA interacts with synthetic chelating agents in the same remarkable way as these interact with each other (Heath and Clark, 1956*a*, *b*).

The evidence that synthetic chelating agents can act as growth substances has already been presented in sufficient detail (Heath and Clark, 1956*a*, Table II), although more information about the assay techniques used is given in the next section of the present paper. To recapitulate briefly: significant increases of growth were given, at concentrations of 10^{-5}M. or less, by the eight synthetic chelating agents tried, viz. α:α-dipyridyl, sodium diethyldithiocarbamate (DIECA), 8-hydroxyquinoline (8HQ), iminodiacetic acid, nitrilotriacetic acid, anthranilic acid-diacetic acid, uramildiacetic acid, and ethylenediaminetetra-acetic acid (EDTA), in every case both in the presence and absence of 10^{-5}M. IAA. The non-chelating analogues, γ:γ-dipyridyl hydrate and 2-hydroxyquinoline (2HQ) at the same concentrations gave either inhibition or no significant promotion of growth (except for an isolated case apparently due to a fortuitously low 'control'). Since DIECA is a heavy metal enzyme inhibitor, its effects were compared at 10^{-9} to 10^{-6}M. with those of KCN which was found to reduce growth in the absence of 10^{-5}M. IAA and be without effect in its presence. The only attributes common to all eight chelating agents, whose very various molecular structure in many cases fails to conform to the above-mentioned 'rules' for auxins, are the possession of nitrogen and the ability to chelate. More recent work (Heath and Clark, unpublished) has shown that sodium oxalate, which chelates but has no nitrogen, can cause significant growth promotion at 10^{-6} to 10^{-9}M.

There seemed no reason to doubt that the growth promotion caused by the various agents was due to chelation and the hypothesis was made that IAA, and possibly other growth substances also, acted in the same way. Dr. E. R. Roberts of the Chemistry Department, Imperial College, considered that IAA should in theory be able to form chelate compounds or complexes with metals and, as mentioned above, more recent work has shown this to be the case, at least for copper and ferric ions, though the latter bring about slow decarboxylation of the IAA (Recaldin and Heath, 1958).

For each of the eight chelating agents tried the greatest growth response found after 24 hours' treatment with any concentration of the agent alone was significantly less than that due to 10^{-5}M. IAA alone; in the case of 10^{-5}M. EDTA there was evidence that this was due to slower penetration, for by 36 hours the growth was practically the same as with 10^{-5}M. IAA. Fawcett *et al.* (1956), however, using a different strain of wheat and different technique, found that EDTA gave much less extension, compared with controls, than

IAA and further that the optimum concentrations were 10^{-3}M. for EDTA and 10^{-4}M. for IAA. They compared the effect of EDTA with the similar extension found using $10^{-2.5}$M. trichloroacetic acid, $10^{-3.5}$M. carboxymethyl o-*tert*-butylxanthate or 10^{-2}M. to 10^{-1}M. ethanol, and considered that these four compounds were 'not acting as true "growth substances" '. They suggested that their action might well 'depend upon changes in membrane permeability and cell-wall structure resulting from sub-acute toxicity', apparently postulating that a relaxation of wall pressure precedes loss of turgor in moribund tissue; the present authors are unaware of any evidence to support this implicit suggestion. Experiments described in the present paper show that the discrepancies between the magnitudes of the EDTA and IAA effects found by Fawcett *et al.* and the present authors are to be attributed partly to the different strains of wheat used but mainly to the differences in technique. Bennet-Clark (1956) found that oat coleoptile sections showed growth responses to 10^{-5} to 10^{-3}M. EDTA considerably smaller than that to 10^{-6}M. IAA. On the other hand, working with sections of etiolated lupin hypocotyl, Weinstein *et al.* (1956) found a response to 5×10^{-5}M. EDTA of the same order as that to 10^{-5}M. IAA. Maximum response to EDTA alone occurred at 5×10^{-5}M. or 10^{-4}M.; with the former concentration, respiration (oxygen uptake) showed a 50 per cent. increase. The question of the theoretical importance of the magnitudes of the effects of chelating agents on growth, as distinct from that of establishing their existence, is considered in the Discussion (p. 17).

More important than the ability of IAA to chelate or form complexes with metals *in vitro* is the question of whether it does so *in vivo*; here the evidence obtained rests upon the interactions of IAA and known chelating agents in experiments on the growth of intact root systems and of coleoptile sections. The results of some of these experiments have been very briefly described in the two preliminary communications cited, and the present paper is devoted mainly to their detailed presentation and discussion.

MATERIALS AND METHODS

All chemicals used were of the purest grade available commercially. Distilled water, from a tinned boiler and Pyrex glass condenser, was redistilled from glass, collected in a Pyrex flask and stored at $+2°$ C. in a polythene bottle for not more than 24 hours before being used to make up stock solutions or to set up an assay. Unbuffered solutions were used throughout in order to avoid salt effects.

Except where otherwise stated the wheat seed used in the experiments described was *var.* Eclipse (1955 harvest). It gave excellent germination and very uniform coleoptiles after 72 hours at 25° C. in total darkness.

Root-growth inhibition method for bioassay of growth substances

The seeds were surface sterilized in 30 per cent. v/v sodium hypochlorite solution for 20 minutes, washed with ten rinses of sterile distilled water, and

soaked in distilled water for 3 hours; they were then transferred in batches of 50 to moist filter-paper in 9·5 cm. diameter petri-dishes, each with 4·0 ml. of sterile distilled water. These were kept in the dark at 25° C. for 20 hours. Seeds with radicles between 1·5 and 2·5 cm. in length were then selected and each was threaded asceptically on to a 6·0 cm. length of stainless-steel wire, bent as in Fig. 1, which had been heated to 120° C. and cooled. After a further 24 hours' development on moist filter-paper as before, the seedlings were selected for uniformity and allotted to the treatments at random. Each was supported in the mouth of a $6 \times \frac{5}{8}$ in. Pyrex glass test-tube, holding 20 ml. of test solution, with the tip of the radicle immersed in the solution (Fig. 1). The tubes were randomized in racks placed in a dark cupboard which maintained a fairly steady temperature of about 20° C. Specimen tubes were inverted over the test-tubes for the first 2 days to keep the humidity high. The cupboard was ventilated with a fan twice a day and once every 3 days each seedling was transferred to a test-tube of fresh solution; apart from this they were kept in total darkness.

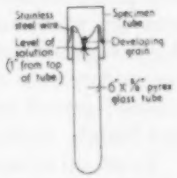


FIG. 1. Root-growth method of assay. Diagram showing assay tube 24 hours after setting up the test.

After 8 or 9 days from germination the roots were cut off and measured to the nearest 0·5 mm., the length of the longest root and total length being recorded for each plant. The two measures were highly correlated and only data for total length of root per plant are presented here.

Straight growth test with wheat coleoptile sections for bioassay of growth substances

The methods used were slightly modified from those described by Hancock and Barlow (1953).

Wheat seed was soaked in distilled water for 3 hours and spread (about 1 per cm.^{2}) on moist medium-grade vermiculite which had been evenly compressed and had enough distilled water added to leave 1 cm. free water at the bottom of the tin used. The seed was covered with 1 cm. of vermiculite and the tins kept in darkness at 24·5° C., generally in a large box ventilated with saturated air. Daily additions of water through a wide glass tube reaching to the bottom of the tin maintained the layer of free water. During this operation and in all other handling of seedlings or coleoptiles, a 'Kodak' safelight with Wratten OA filter was used to avoid risk of phototropic curvature (Heath, 1952). About 40 per cent. of the seeds sown gave uniform coleoptiles of a suitable length after 72 hours under these conditions.

Coleoptiles of the size grade 18–20 mm. at 72 hours were used throughout this work; 1 cm. sections 3 mm. back from the tip were cut and the first leaf was not removed from the section.

In preliminary experiments all the coleoptile sections were placed on wet filter-paper and were allotted to the treatments at random after the whole batch needed for an experiment was cut. Later, and in all the experiments here described, each group of five coleoptile sections cut was immediately placed

in the test solution for one of the treatments, chosen at random, at a noted time. This was found to reduce the standard deviation for one 'replication' (the mean of five coleoptile sections) from about 0·05 cm. to 0·02 cm., or 1 per cent. of the mean length after 24 hours' growth. An effect somewhat resembling this was obtained by Brian *et al.* (1958) who found an improvement in accuracy when using 'unwashed' instead of 'washed' pea epicotyl sections. Some trials of washing the coleoptile sections for 1 hour in distilled water before they were allotted to the test solutions showed reduction neither in the 'control' growth nor in the variability (cf. Fransson and Ingestad, 1955; Nitsch and Nitsch, 1956); this preliminary washing was therefore abandoned.

To set up an assay, 0·5 ml. aliquots of the test solutions were pipetted into $2 \times \frac{3}{8}$ in. specimen tubes. As each group of five coleoptile sections was placed in one of these it was shaken, corked with a freshly waxed cork and placed horizontally in a metal container on a large klinostat rotating at about 12 revolutions an hour.

After 24 hours the sections were removed and measured to the nearest 0·5 mm., the tubes being taken in the same random order as when they were set up.

It may be mentioned that a feature of this technique is that it gives a great deal of growth in distilled water, small responses to growth substances but very low variability and hence high precision in treatment comparisons. The large amount of 'control' growth is of course advantageous for the investigation of inhibitors, or concentrations of growth substances causing inhibition.

EFFECTS OF ASSAY TECHNIQUE AND STRAIN OF WHEAT

Fawcett *et al.* (1956) found the greatest response of wheat coleoptile sections to EDTA at 10^{-3}M. concentration, in which the length after 24 hours was about 18 per cent. greater than that of the water 'controls', while the optimum concentration of IAA (10^{-4}M.) gave a length nearly 80 per cent. greater than the controls. Presentation of all data as percentages of 'control' length renders comparisons difficult, for it not only makes it impossible to judge the variability between different experiments but obscures the actual amount of extension growth made. It appears, however, that the amount of 'control' growth was much less in Fawcett *et al.*'s experiments (personal communication) and the main discrepancies in the published results were as follows:

	Heath and Clark	*Fawcett et al.*
10^{-3}M. EDTA	Optimal and almost as effective as 10^{-5}M. IAA	Without effect
10^{-2}M. EDTA	Severely inhibitory	Optimal but much less effective than 10^{-5}M. IAA

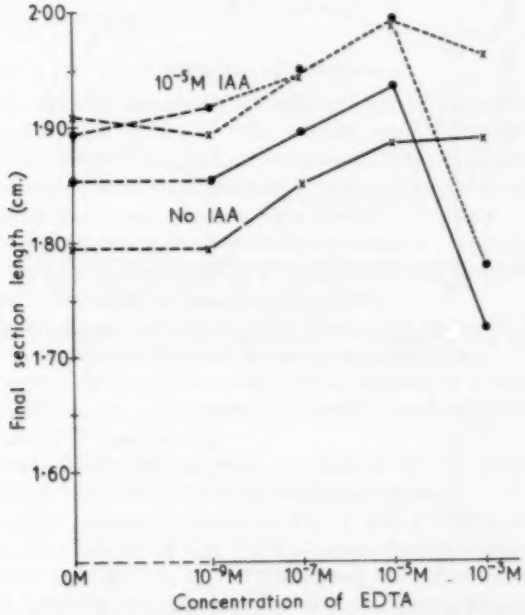
A series of experiments (Figs. 2–4[1]) was therefore carried out to compare

[1] Owing to the fact that the corresponding curves for treatments with and without IAA are nearly parallel, the 3-factor interactions indicated in Figs. 2–4 fail to reach statistical significance except for the experiment of 17.10.56 (Fig. 3), which had a particularly low error

the chemicals, strain of 'Eclipse' wheat, and technique used by Fawcett *et al.* with those used in the rest of the investigations.[1]

Experiment of 5.10.56. Comparison of chemicals and strains of wheat

This was a factorial experiment of 40 combination treatments, viz.: 5 concentrations of EDTA (O M., 10^{-9}M., 10^{-7}M., 10^{-5}M., 10^{-3}M.)$\times$2 concentrations of IAA (O M., 10^{-5}M.)$\times$2 sources of chemicals (Wye College supply, Imperial College supply)$\times$2 strains of 'Eclipse' wheat (Wye College supply, Imperial College supply); for each treatment there were 2 replicates of 5 coleoptile sections each; the technique used was the usual one as described in the previous section.



FIGS. 2–4. Comparison of coleoptile straight growth assay methods.
FIG. 2. Imperial College technique using Wye College wheat (–x–) and Imperial College wheat (–•–).

Statistical analysis showed both the main effect of 'source of chemicals' and all interactions involving this factor to be entirely non-significant. This was satisfactory as an indication that the discrepancies were not attributable to impurities in the chemicals used. The means for the other treatments, averaged

variance. The diagrams are nevertheless given in this form in order to show the average IAA effects.

[1] We wish to thank Professor R. L. Wain for kindly supplying chemicals, seed, and details of the technique used at Wye College.

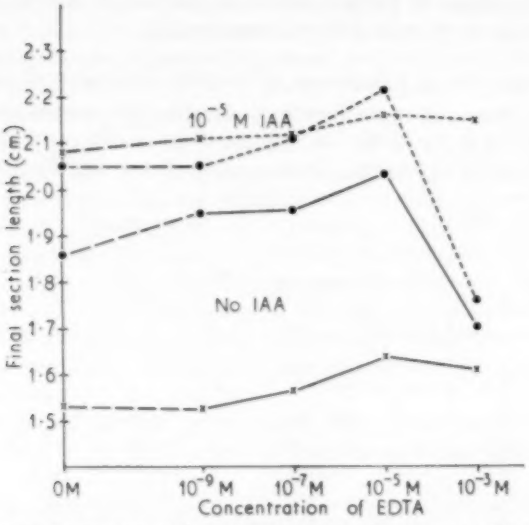


FIG. 3. Comparison of Wye College (–x–) with Imperial College (–•–) routine methods using Imperial College wheat.

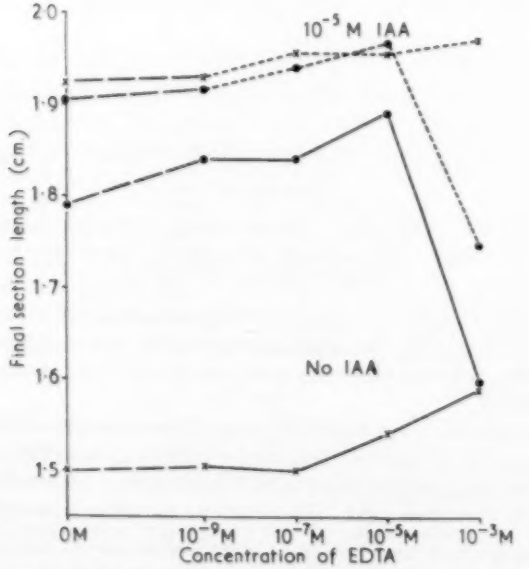


FIG. 4. Comparison of Wye College technique and seed supply (–x–) with Imperial College technique and seed supply (–•–).

over both sources of chemicals, are presented in Fig. 2, which shows that the discrepancy as to the optimal concentration of EDTA could be attributed to the strain of wheat used, severe inhibition occurring at 10^{-3}M. with the usual supply but growth increasing up to this level (in the absence of IAA) with the strain from Wye. It is also seen that the 'control' growth was slightly less for the Wye wheat, and since the growth with IAA alone was about the same, the response to IAA was greater for the Wye wheat. The response to EDTA (up to 10^{-5}M.) was almost the same for the two strains, both in the absence and in the presence of IAA.

Experiment of 17.10.56. Comparison of techniques

This was a factorial experiment of 20 combination treatments, viz.: 5 concentrations of EDTA (O M., 10^{-9}M., 10^{-7}M., 10^{-5}M., 10^{-3}M.)$\times$2 concentrations of IAA (O M., 10^{-5}M.)$\times$2 techniques (Wye College and Imperial College); for each treatment there were 2 replicates of 5 coleoptiles each; the usual chemicals and strain of wheat were used.

The chief differences in the Wye technique were:

1. After the coleoptile sections were cut the first leaf was pushed out completely, using a blunt-ended glass capillary tube.
2. The sections were then washed by floating on water for 1 hour.
3. They were then threaded on to fine glass capillaries.
4. They were then floated on 10 ml. of test solution in a covered petri-dish (compared with 0·5 ml. of solution in a small corked tube in the technique used at Imperial College).
5. The petri-dishes were kept in the dark at 25° C., with the coleoptile sections floating horizontally (but not of course rotated).

The treatment means are shown in Fig. 3, where it appears that the discrepancy in the effect of 10^{-3}M. EDTA could also be largely attributed to the difference of technique, in the absence of the effect of strain of wheat noted in the previous experiment. The Wye technique also resulted in very much less control growth ($P<0{\cdot}001$) than the usual technique but about the same growth with IAA alone; 'response' to IAA was therefore greater. Thus far the differences due to technique were very similar to those due to strain of wheat. The response to EDTA was somewhat smaller with the Wye technique.

The next experiment was designed to investigate the combined effects of technique and seed, thus simulating those carried out at Wye and Imperial College respectively.

Experiment of 25.10.56. Comparison of Wye technique and seed with Imperial College technique and seed

This was a factorial experiment of the same design as the previous one except that Wye seed was used with the Wye technique and the usual seed for the Imperial College technique.

As may be seen in Fig. 4, the pattern of results was similar to that in the two previous experiments, the effects of seed and technique reinforcing each

other. The 'Wye' control growth was very much smaller and hence the 'response' to IAA much greater, since the amount of growth in IAA alone was almost identical. The maximum response to EDTA was almost the same in both cases, but occurred at 10^{-3}M. for 'Wye' and 10^{-5}M. for 'Imperial College'. Thus the principal differences lay in the amount of control growth, the response to IAA and the optimal concentration of EDTA. Expressed as percentages of the control length the results for EDTA or IAA alone were:

	'Wye'	'Imperial College'
Optimal EDTA	6·0 (10^{-3}M.)	5·6 (10^{-5}M.)
10^{-5}M. IAA	28·7	6·7

The corresponding figures obtained by Fawcett *et al.* were 18 for 10^{-3}M. EDTA and 66 per cent. for 10^{-5}M. IAA.

It would thus appear that the discrepancies between the results of Fawcett *et al.* and the present authors were due in the main to the different techniques and strains of wheat used. A very considerable research would be needed to elucidate the precise aspects of the two techniques responsible for the striking differences observed.

A number of further experiments was in fact carried out to investigate effects both of variations in technique and of different varieties of wheat. These gave further evidence that amount of control growth, response to IAA and values of optimal concentrations could be considerably modified. For instance, an experiment on the effects of removing the first leaf showed that this much reduced control growth ($P<0{\cdot}001$) and hence doubled the response to 10^{-5}M. IAA; the response to 10^{-5}M. EDTA was little affected, but 10^{-3}M. caused further slight increase in growth if the first leaf was removed and not if it was left in position. The series of experiments was, however, too incomplete to justify presentation in detail here.

INTERACTIONS OF IAA AND KNOWN CHELATING AGENTS

(*a*) *Effects on root growth* (*intact seedlings*)

The technique described on p. 170 was used throughout, as was also the usual strain of Eclipse wheat except for the preliminary experiment described below. The solutions were made up in distilled water without nutrients.

Preliminary experiment of 10.1.56 with wheat var. Holdfast. Interaction of IAA and EDTA

This was a factorial experiment of 16 combination treatments, viz.: 4 concentrations of IAA (O M., 10^{-9}M., 10^{-7}M., and 10^{-5}M.) × 4 concentrations of EDTA (O M., 10^{-9}M., 10^{-7}M., and 10^{-5}M.); for each treatment there were 2 replicates of 5 seedlings.

The treatment means and least significant differences are shown in Table I. The striking result is seen that although, as expected, either IAA or EDTA alone at 10^{-5}M. concentration caused severe inhibition of root growth, this

inhibition was removed by the presence of 10^{-7} M. (i.e. one-hundredth the amount) of the other agent. With both agents at 10^{-5} M. the inhibition was again severe.

TABLE I

Experiment of 10.1.56. Length of roots (cm.) of wheat seedlings (var. Holdfast) after 8 days' growth.

Means of two replicates each of 5 plants. Significant differences for two means of two replicates ($n = 16$) P 0·05 = 2·47; 0·02 = 3·01; 0·01 = 3·41; 0·001 = 4·68.

Concentration of IAA	Concentration of EDTA			
	O M.	10^{-9}M.	10^{-7}M.	10^{-5}M.
O M.	23·00	23·15	20·50	3·02
10^{-9}M.	21·90	24·60	19·30	6·00
10^{-7}M.	18·15	10·04	18·80	22·00
10^{-5}M.	0·80	6·65	21·50	5·35

Experiments of 22.3.56 and 21.4.56. Interaction of IAA and EDTA

These two factorial experiments were of identical design, viz.: 25 treatments given by IAA and EDTA at O M., 10^{-11}M., 10^{-9}M., 10^{-7}M., and 10^{-5}M., in all possible combinations; for each treatment there were two replicates of three seedlings. The treatment means and least significant differences are shown in Fig. 5. EDTA alone and IAA alone again caused severe inhibition of root growth at 10^{-5}M., but the strange mutual antagonism seen in the preliminary experiment was here even more striking, for a concentration of 10^{-11}M. of either agent was sufficient to remove practically the whole of the inhibition due to a 10^{-5}M. concentration of the other. With wheat of *var.* Eclipse, therefore, only one millionth the concentration of EDTA was needed to remove the ill effects of 10^{-5}M. IAA and vice versa. Once again, with both agents at 10^{-5}M., inhibition was severe. The slight apparent stimulation of root growth with 10^{-11}M. concentration, which has often been claimed for IAA, in all cases failed to reach significance.

In view of this remarkable interaction it was decided to try two known chelating agents in a similar experimental design.

Experiments of 4.5.56, 24.5.56, and 7.6.56. Interaction of EDTA and 8HQ

The design was identical with that of the two preceding experiments but with 8HQ substituted for IAA. The treatment means and least significant differences are presented in Fig. 6. Although the results of one of the experiments (4.5.56) were somewhat irregular and the errors large, the resemblance of the interaction to that of EDTA and IAA (Fig. 5) is very close and the same mutual antagonism is shown.

Experiment of 20.6.56. Interaction of EDTA and 2HQ

This was identical with the foregoing but with the non-chelating analogue 2HQ substituted for 8HQ. The results are shown in Fig. 7, where it is seen

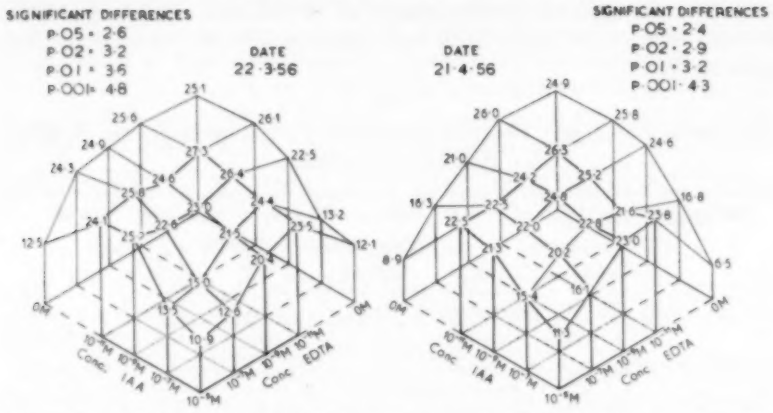


FIG. 5. The effects of IAA and EDTA on root growth. Two experiments—using wheat (*var.* Eclipse). Measurements are in cm.

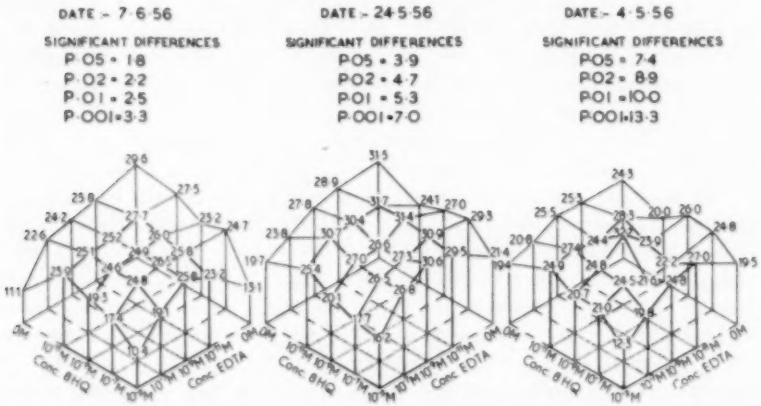


FIG. 6. The effects of EDTA and 8HQ on root growth. The results of three experiments are given. Measurements are in cm.

that the roles of the two agents are no longer interchangeable. The inhibition due to 10^{-5}M. EDTA alone was in fact reduced in the presence of 10^{-11}M. 2HQ, but the less drastic inhibition caused by 10^{-5}M. 2HQ alone was much more severe in the presence of 10^{-11}M. EDTA.

Experiments of 2.7.56, 12.7.56, 23.7.56, and 14.11.56. Interaction of IAA and 2HQ

These experiments were of the same design as the foregoing but with IAA instead of EDTA. The first of them (2.7.56) showed a mutual antagonism similar to that found with IAA and EDTA. This appears to have been an

aberrant result, for none of the other experiments using the same agents showed any tendency for a 10^{-11}M. concentration of one agent to prevent the inhibition caused by a 10^{-5}M. concentration of the other. The most satisfactory course seemed to be to carry out a combined analysis on all four experiments, and this gave no indication of a significant mutual antagonism; in fact the interaction variance was no larger than the error variance. The treatment means for the four experiments are presented in Fig. 8.

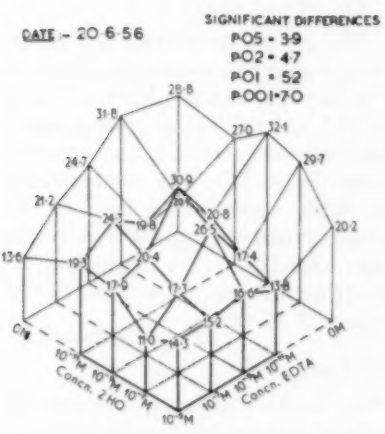


FIG. 7

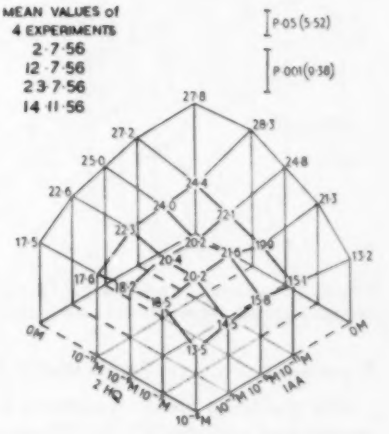


FIG. 8

FIG. 7. The effects of EDTA and 2HQ on root growth. Measurements are in cm.
FIG. 8. The effects of IAA and 2HQ on root growth. Measurements are in cm.

(*b*) *Effects on coleoptile section growth*

In view of the remarkable effects found for root growth, a series of factorial experiments on coleoptile growth was carried out including supra-optimal concentrations of the agents in order to obtain inhibitory effects. The design of experiments with pairs of agents was the same as that used for the root-growth experiments but with all concentrations multiplied $\times 100$. Methods used were as described above (p. 171).

Experiment of 12.6.56. Interaction of IAA and EDTA

The results of this experiment are shown in Fig. 9. The same mutual antagonism is seen as in the root-growth experiments with these agents, but at intermediate concentrations (up to 10^{-5}M.) stimulation occurred.

Experiment of 17.7.56. Interaction of IAA and 8HQ

The results of this experiment, given in Fig. 10, much resemble those of the experiment with IAA and EDTA (Fig. 9).

Experiment of 4.7.56. Interaction of IAA and 2HQ

Here the non-chelating 2HQ showed inhibitory effects throughout and either agent at 10^{-9}M. somewhat increased the inhibition caused by a 10^{-3}M. concentration of the other (Fig. 10).

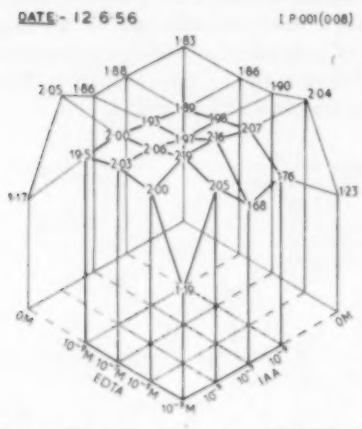


FIG. 9. The effects of IAA and EDTA on coleoptile growth. Measurements are in cm.

Experiment of 27.6.56. Interaction of EDTA and 8HQ

The results, presented in Fig. 10, show a mutual antagonism similar to that between IAA and EDTA (Fig. 9) or IAA and 8HQ (Fig. 10) but somewhat less symmetrical; the improvement due to a 10^{-9}M. concentration of either agent added to a 10^{-3}M. concentration of the other is highly significant ($P<0.001$) although rather small in the case of 10^{-9}M. EDTA with 10^{-3} M. 8HQ.

Experiment of 9.7.56. Interaction of EDTA and 2HQ

The treatment means, presented in Fig. 10, show a slightly asymmetrical interaction, with 10^{-9}M. EDTA removing a little of the inhibition due to 10^{-3}M. 2HQ and 10^{-9}M. 2HQ increasing slightly the inhibition due to 10^{-3}M. EDTA, i.e. the reverse of the roles shown in the root-growth experiment of 20.6.56.

Experiment of 7.11.56. Interaction of 8HQ and 2HQ

This experiment gave no indication of removal of the inhibition due to high concentration of 8HQ by a low concentration of 2HQ (Fig. 10).

DISCUSSION

The most important findings in the present work are thought to be those relating to the mutual antagonism between IAA and known chelating agents and to the similar antagonism between pairs of the latter, as presented in the previous section. It is proposed to discuss these interactions first, then to mention other evidence supporting the hypothesis that IAA acts in the plant by chelation or complex formation and finally to consider various criticisms that have been levelled against this hypothesis.

The interaction between IAA and EDTA, whether as affecting root growth or coleoptile growth, is on the whole remarkably symmetrical (Figs. 5 and 9) and the names of the two agents could be interchanged without appreciably affecting the conclusions reached. Either agent alone in 'high' concentration

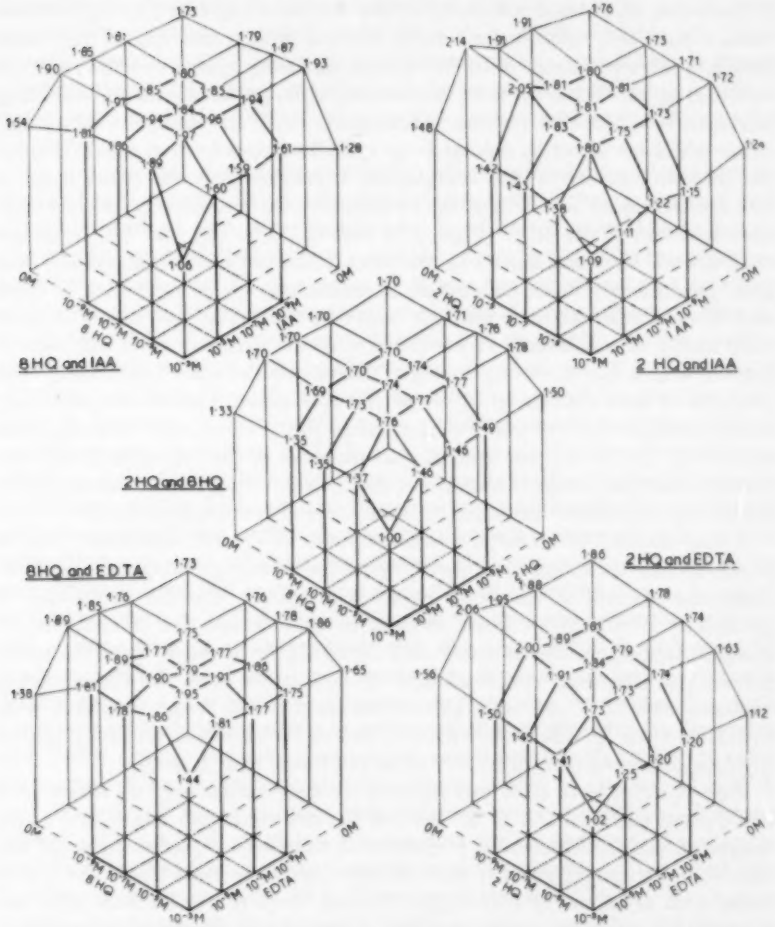


FIG. 10. The effects of various mixtures of agents on coleoptile extension. Measurements are in cm. Significant differences: 8HQ+IAA, 8HQ+2HQ, 2HQ+IAA, 8HQ+EDTA, P 0·001 = 0·08; 2HQ+EDTA, P 0·001 = 0·07.

severely inhibits growth, but this inhibitory effect is almost or entirely removed by the presence of one millionth the concentration of the other. When both agents are present in 'high' concentration inhibition is again severe. This interaction most strongly suggests that the mode of operation of the two agents in the plant is similar, but it is clear that their action cannot be *identical*, in the sense that one can substitute for the other in all combinations of concentrations, for the addition of, say, 10^{-11}M. IAA to 10^{-5}M. IAA could scarcely prevent inhibition. Experiments with IAA and 8HQ or with 8HQ and EDTA

(Figs. 6 and 10) showed essentially similar mutual antagonism, though in some cases rather less symmetrical. Such mutual antagonism has in fact been found in all experiments with two known chelating agents or with IAA and one such agent—it has not been found in experiments in which a non-chelating analogue is substituted for one of the agents (with the exception of a single result which could not be repeated—p. 178). Such substitution either destroys the mutual character of the antagonism or entirely prevents either agent at low concentration from reducing measurably the inhibition caused by high concentration of the other (Figs. 7, 8, and 10). The fact that IAA interacts with known chelating agents in the same remarkable and symmetrical way that these interact with each other, in controlling the growth both of roots and of coleoptile sections, strongly supports the hypothesis that it is itself here acting as a chelating or complexing agent; the absence of such interactions with a non-chelating analogue makes this the more convincing. The problem of how a single molecule of one species can remove the inhibition due to a million (or even only a hundred as in the experiment with Holdfast wheat—p. 176) of another species, and the roles of the two species be completely interchangeable, is obscure in the extreme. Speculation has not so far led to any hypothesis for which an experimental test suggests itself.

These results are concerned with removal of growth inhibition, due to supra-optimal concentrations of one agent, by minute proportions of another molecular species; it cannot be regarded as proven that the promotion of growth by IAA in the absence of another agent is also due to chelation or complex formation. Nevertheless, this seems highly probable when the interactions just discussed are considered in conjunction with the accumulating evidence that IAA and other growth substances can form complexes with metals *in vitro* (p. 169) and with the finding that of nine chelating agents tried all promoted growth in the wheat coleoptile test (p. 169).

Two theories have been put forward for the possible way in which IAA could form chelates. Dr. E. R. Roberts (quoted by Heath and Clark, 1956) suggested the formation of a 7-membered ring with chelation between the imino- and carboxyl groups; such chelates would be most stable if a heavy metal such as copper or cobalt was involved. Even if true chelates were not formed Roberts expected complex-formation to occur. Experimental evidence of complex-formation or chelation was later obtained by Cohen *et al.* (1958) and by Recaldin and Heath (1958); the former authors found that α-napthylacetic acid could also form complexes, and as this lacked a replaceable hydrogen on a basic atom in the ring they postulated that the entire aromatic ring functioned as the second complexing group, both in this growth substance and in IAA. In support of this they cited reports of complex formation between metal ions and various aromatic compounds (Anet and Leblanc, 1957, and Ogimachi *et al.*, 1956, cited by Cohen *et al.*, 1958). Armarego *et al.* (1959) preferred for 2:4-dichlorophenoxyacetic acid (2:4-D) the hypothesis that complexing occurred between the two acid oxygen atoms. These authors consider that the hypothesis that growth substances act by chelation or complex

formation is disproved by their observation that the stability constants of copper complexes are almost the same for 2:4-D, which is highly active as a growth substance, and for 2:4-dimethylphenoxyacetic acid, which is almost inactive; they further point out that both these and the stability constants of the copper complexes formed by α-naphthylacetic acid and IAA are of a low order.

To suppose that the growth-promoting effects of chelating or complexing agents should depend solely on the stability constants of their complexes with copper (or indeed with other metals also) would seem to be demanding an excessively simple biological system, and in putting forward the hypothesis in question (Heath and Clark, 1956*a*, *b*) it was not suggested that no other properties of the molecules concerned could modify their effects on growth. In the case of EDTA evidence was obtained that its slightly smaller growth-promoting activity than IAA, as shown after 24 hours, might well be due to slower penetration, for after 36 hours no difference could be demonstrated. Even so, however, its *in vitro* activity as a chelating agent is of course enormously greater than that of IAA and in general the growth-promoting activities of the nine chelating agents tried in no way ran parallel to their metal-chelating capacities. Not only may the very various molecules have numerous side-effects which may modify the amount of growth promotion obtained but steric hindrance may result in the effective chelating activity *in vivo* being very different from that *in vitro*. It is in fact most surprising that so powerful a chelating agent as EDTA does not disrupt the chemical organization of the cell at many sites; it seems probable that this may be due in part to steric hindrance and also that the growth promotion obtained may be the net result of stimulatory and inhibitory processes, perhaps due to competition with various internal chelating agents in some such way as suggested by Burström and Tullin (1957). For such reasons the actual magnitudes of the growth-promoting effects of synthetic chelating agents, which in the case of EDTA have been found to vary greatly with different plant material and different techniques (pp. 172–6), seem less important, at the present stage of investigation, than the fact that they are found in so many instances. The rather low stability constants found for IAA (Cohen *et al.* and Recaldin and Heath give 5·5 and 3·0 respectively as the value of log K for copper) may be advantageous for growth regulation, since IAA would thus only be expected to form complexes with metal ions when very close to them in high effective concentration (e.g. on a surface), avoiding in this way the risk of wholesale disorganization of metabolism.

It has already been noted that the mode of action of EDTA cannot be *identical* with that of IAA (p. 181), and it has been found inactive in the mesocotyl growth test (Thimann and Takahashi, 1958) as well as in the pea curvature and tomato leaf epinasty tests (Fawcett *et al.*, 1956); in view of the considerations just put forward, however, it by no means follows from this that 'chelation cannot be a universal mechanism leading to all types of growth response' as stated by Fawcett *et al.*, although this has not in fact been

claimed. Burström and Tullin (1957) working with EDTA in culture solutions (where it was presumably chelated with the $^{++}$Mg or other bivalent metals supplied) found that it reduced root growth by effecting cell division but not cell extension; for IAA they stated the converse to hold. This observation may well be correct, but it is not apparent that it elucidates the interactions which have been found here between chelating agents and IAA supplied in the absence of metal ions. They were unable to obtain consistent results under the latter conditions and state that distilled water is not a reproducible medium for root-growth experiments.

The discrepancies between the results of Fawcett *et al.* and the present authors raise the question of choice of techniques and material when using biological assay methods in the attempt to elucidate the mechanism of action of known compounds, as in the present paper. In such work the choice must be made between carrying out an extensive investigation with many biological tests, or a more intensive one in which one or a few tests are employed. The latter procedure makes it possible to carry out factorial experiments and thus investigate interactions between compounds. It is legitimate, and may be advantageous in the first instance, to try to elucidate a mechanism in a very restricted field; for instance, using a single type of response even on a single individual plant or, what is nearly the same thing, on a single clone. If this is done, however, the results may be of very limited application and must be checked over a wider field. An example is provided by the striking differences found by Harris (1959) in the responses to amino-acids of two clones of excised red-clover roots. In the present work, carried out mainly with a single supply of wheat, the population was presumably more diverse than in a clone; moreover, two different types of response were employed (i.e. root growth on intact plants and growth of coleoptile sections) and found to give strikingly similar results. Nevertheless, large quantitative differences were found to exist between varieties (Table I and Fig. 5) and strains (Fig. 2) as was perhaps to be expected; qualitative differences would have been more serious but did not appear. Again, the consistent and remarkable qualitative form of the interaction between pairs of agents seems far more important than the magnitudes of the responses to different agents in different strains.

Large and as yet unexplained differences in responses have also been found with variations in technique, but these differences also were mainly in magnitude of response and effective concentration range. In particular, with no technique or strain was any difference observed between the appearance of coleoptile sections which had been grown in EDTA solutions and of those which had been in IAA solutions of the same strength; there was certainly no similarity in the external appearance of either with that of sections treated with 1×10^{-2}M. ethanol as used by Fawcett *et al.* It is felt that there is no support from the present work for their view that the growth-promoting effect of EDTA is due to 'sub-acute toxicity' (see p. 170).

In view of the large amount of 'control 'growth given by the coleoptile-section technique used for most of this work, it might be suggested that little

further growth was possible and that this would account for the approximate equality of the IAA and EDTA effects obtained, each agent causing the greatest possible extension. This is negatived by the fact that 10^{-5}M. EDTA added to 10^{-5}M. IAA gave further growth almost equal to the increase it caused in the absence of IAA (e.g. Figs. 2–4). The much smaller 'control' growth given by the Wye technique may well be in large measure due to the removal of the first leaf from the section; the results of Hancock and Barlow (1953) and of Bentley and Housley (1954) show that when the leaf is left in position better growth occurs than when it is placed separate in the solution or removed completely (see also p. 176). This suggests either that the coleoptile is injured by the removal of the leaf, though in this case the effect of the injury would rather appear to be overcome by IAA (Fig. 3), or that the leaf produces a substance, possibly an auxin, which stimulates the extension of the coleoptile only when it is in close contact with the leaf—this might be a concentration effect. If this substance were IAA, resulting in a high endogenous concentration with the usual technique but not with the Wye technique, the discrepancies might be explained as due to a synergistic effect between EDTA and IAA, the former giving relatively little growth promotion in the absence of the latter. A brief report from Thimann and Takahashi (1958) states that EDTA and 8HQ promote growth of oat coleoptile sections (but not mesocotyl sections) and that these effects are much greater in the presence of IAA. They suggest that the chelating agents act as synergists and not as true auxins. The data presented in Figs. 3 and 4, however, give no evidence of synergism, the response to EDTA with the Wye technique being less in the presence than in the absence of IAA. Other aspects of technique also make surprisingly large differences to the amount of 'control' growth and response to IAA—e.g. whether the sections are rotated or not (Hancock and Barlow, 1953)—and at present there seems little possibility of explaining such differences.

If it is accepted that IAA does regulate growth by virtue of its chelating or complex-forming properties, and all the evidence in the present work is consistent with this, there is a variety of ways in which it might function. Perhaps the most attractive hypothesis is that it increases plasticity of the cell wall by removing calcium from the pectic components, as suggested for the effect of EDTA by Bennet-Clark (1956) and for IAA itself by Heath and Clark (1956*a*) and Ginzburg (1958); it should be noted, however, that no *in vitro* evidence has yet been obtained for chelation or complex-formation between IAA and calcium (Cohen *et al.*, 1958; Recaldin, unpublished), and although this does not prove the negative, chelation with a heavy metal would seem more probable. Glasziou (1957) suggested that IAA participated in the bonding of pectin methylesterase to the cell wall. This would lower the activity of the enzyme in the cell and so decrease the rate of hydrolysis of pectins, which would accumulate. This agrees with Bennet-Clark's (1956) suggestion that methylation of pectins results in plastic extension of the wall under the influence of turgor. Alternatively, IAA might act as an enzyme inhibitor, either by masking

functional heavy metal ions in prosthetic groups or by eliminating metals needed as coenzymes in some enzyme-controlled system. Smith, Lumry, and Polglaze (cited by Martell and Calvin, 1951) showed that the Mg-stimulated enzyme carboxypeptidase was strongly inhibited by IAA. An indication of a possible relation between IAA and a copper-enzyme arises from the work of Ostravaskaya (1956) who found that germination of rice seeds with a low copper content was stimulated by IAA whereas there was no similar effect with copper-rich seed. Gross (1953) found that a chelating agent (EDTA) in low concentration protected the enzyme adenosinetriphosphatase from traces of heavy metal, and IAA might stimulate enzyme activity in some such way. A final suggestion arises from findings (Calvin (1938) and Weller and Mills (1953), cited by Heath and Clark (1956*a*)) which show that a complex of an acetate and two quinoline molecules with copper can transfer hydrogen and thus act as a catalyst for the hydrogenation of suitable acceptors; IAA might form complexes which act as 'carriers' in a similar way. If IAA could function in two or more of these ways, its wide range of known effects on growth and development would be less surprising. If synthetic growth substances in general also act by complex formation this would account for the similar range of effects produced by molecules of very various structure, ranging from 2:4-D and α-naphthylacetic acid (both shown to form complexes) to α:α-dipyridyl among ring compounds and for non-ring compounds from EDTA to DIECA and carboxymethyldimethyldithiocarbamate (Fawcett *et al.*, 1956; Gordon and Moss, 1958) or even ethylene, which also can form metal complexes.

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The Auxin Activity Extractable from Excised Tomato Roots by Cold 80 per cent. Methanol

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SUMMARY

Extracts of excised tomato roots prepared with cold aqueous methanol have been partitioned between ethyl acetate and water and chromatograms of the two fractions bioassayed by an *Avena* coleoptile straight growth test and sprayed with various reagents including those giving colour reactions with indole compounds. The greater part of the auxin activity is in the aqueous fraction and chromatograms of this fraction give positive indole reactions.

The aqueous fraction chromatographed with isopropanol/ammonia/water shows two zones of growth-promoting activity. The zone of lower Rf contains tryptophane and unidentified ninhydrin-positive material. Tryptophane accounts for the activity of the Ehrlich-positive region of this zone. The activity of the zone of higher Rf is associated with ninhydrin-positive material; this activity can, by use of other solvents, be distinguished in Rf from that of the common amino-acids which would occur in this zone on the primary chromatograms. Chromatograms of the aqueous fraction also show the presence of urea and of a phenol, neither of which are associated with the zones of growth-promoting activity.

The ethyl acetate fraction, and particularly the acid fraction of this, contains growth-promoting activity which, in a range of solvents, always corresponds in Rf to IAA. The acid fraction contains a growth inhibitor corresponding in Rf to the β-inhibitor of other workers. A zone of growth-promoting activity near the solvent front on isopropanol/ammonia/water chromatograms contains more than one active component, but IAN appears to be absent.

INTRODUCTION

CHROMATOGRAPHY of alcoholic extracts of roots after removal of the substances preferentially soluble in ether or ethyl acetate as against water has revealed the presence of zones whose eluates are highly active in *Avena* coleoptile straight growth tests (Britton, Housley, and Bentley, 1956; Audus and Gunning, 1958). By contrast, the ether-soluble material has been of low auxin activity. Positive indole reactions do not seem to have been obtained on chromatograms from such root extracts.

Following upon the suggestions of Audus and Shipton (1952) and of Street (1954), these investigations of the natural auxins in root extracts appeared to justify the hypothesis that root growth is controlled by a non-indole system. This prompted us to re-examine the auxin activity of extracts of excised tomato roots, particularly the preferentially water-soluble auxin activity and

the indications of the interconvertibility of its components on rechromatography (Britton, *et al.* 1956, Audus and Gunning, 1958).

MATERIALS AND METHODS

Culture and extraction techniques. The excised tomato roots were derived from a clone of Sutton's Best-of-All. Penicillin culture flasks containing 1 l. of a modified White's medium (White, 1943; Hannay and Street, 1954) of initial pH 4·8 and containing ferric ethylenediaminetetraacetate as iron source (Sheat, Fletcher, and Street, 1959) were inoculated with 20 root tips (each 20 mm.) and incubated at 27° C. for 28 days. The freshly harvested roots were washed with tap water, surface dried with blotting-paper, weighed, and rapidly cooled to −15° C. They were then macerated in a bottom drive blender in 80 per cent. 'Analar' methanol initially at −15° C. The brei was immediately returned to −15° C. and stored in the dark at this temperature for 24 hrs., filtered, and the methanol removed under vacuum at 27° C. to give an aqueous residue of pH 5·5. The aqueous residue was then adjusted to pH 3·0 with sulphuric acid and shaken four times with twice its own volume of 'Analar' ethyl acetate, keeping each of the four ethyl acetate extracts separate where the total *ethyl acetate fraction* was to be separated into *neutral* and *acid* fractions. This separation was achieved using a bicarbonate countercurrent technique as described by Wright (1956). The *aqueous fraction*, after the ethyl acetate extraction, was adjusted to pH 5·0 by barium hydroxide before chromatography.

Chromatography. Whatman No. 3 MM. paper was used. Chromatographic solvents and location reagents were prepared according to Smith (1958). Chromatograms were dried in a current of cold air or, as with butanol/acetic/water chromatograms, by heating to 30° C. for half an hour. 3-indolylacetic acid (IAA) and 3-indolylacetonitrile (IAN) and in some cases L-tryptophane and urea were used as marker spots. Chromatograms for bioassay were not examined in ultraviolet light. The tryptophane content of the aqueous fraction was estimated by the method of Undenfriend and Peterson (1957). For these determinations the aqueous fraction was not chromatographed directly but first depleted of salts and sugars using Dowex 50 × 8 by the method of Plaisted (1958). The eluate from the column was chromatographed in isopropanol/ammonia/water and the tryptophane located by a marker spot. The tryptophane-containing zone of the chromatogram was then cut into small pieces and eluted directly with 0·5 per cent. *p*-dimethylaminobenzaldehyde in 12N.HCl. Tryptophane standards were prepared by identical elution. Optical densities were read at 625 mμ in a Unicam Spectrophotometer using 1 cm. cells.

Bioassay. Victory oats were grown in the dark in vermiculite at 27° C. for approx. 72 hrs., being exposed to red light from 56–58 hrs. to suppress mesocotyl growth. Sections 4 mm. long were cut 3 mm. below the apex with Thimann's modification of the van der Weij microtome (Nitsch and Nitsch, 1956). Strips of chromatography paper 2·5 × 2·5 cm. were eluted with 2 ml.

pH 5·0 buffer (K_2HPO_4, 1·794 g./l., citric acid 1·019 g./l.) containing 2 per cent. sucrose in 10 ml. Pyrex micro-beakers. Ten *Avena* sections were added and the beakers gently agitated for 24 hrs. in darkness at 27° C. on a Conway

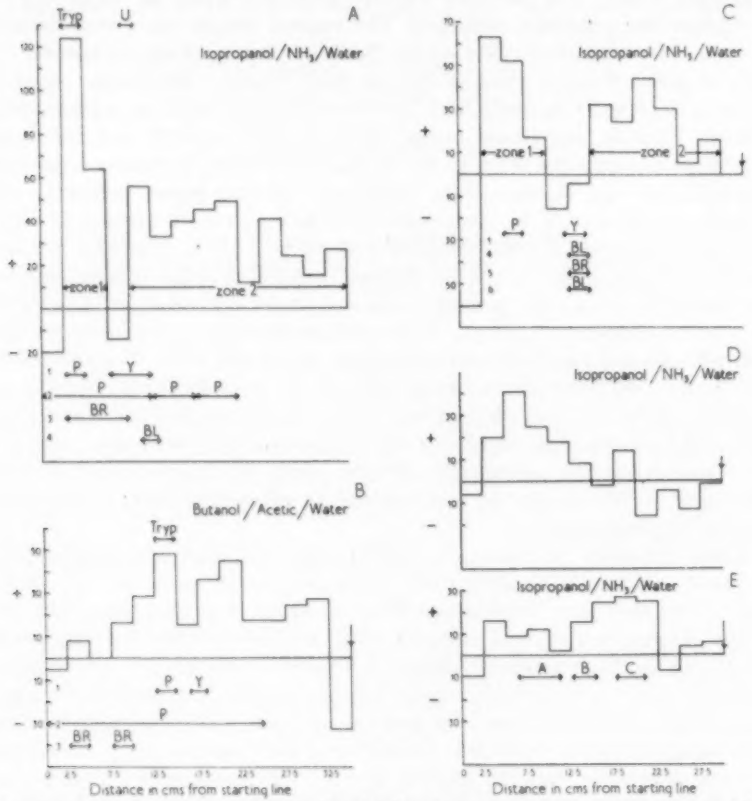

FIG. 1. Chromatograms of the *aqueous fraction* showing colour reactions and growth activity of eluates. Histograms of the growth activity show enhancement (+) and retardation (−) of *Avena* coleoptile extension growth expressed as a percentage of the increase in length of control sections. Key: Tryp. = position of tryptophane marker spot, U = urea marker spot. 1 = Acetone-Ehrlich, P = purple, Y = yellow, 2 = Ninhydrin-pyridine, 3 = Aniline oxalate, BR = Brown, 4 = U.V. fluorescence, BL = blue, 5 = sulphanilic acid, 6 = *p*-nitraniline.

Fig. 1A and B. Aqueous fraction = 2g. fresh wt. root.

Fig. 1C. Aqueous fraction = 4g. fresh wt. root.

Fig. 1D. Eluate from Zone I of chromatogram 1C.

Fig. 1E. Eluate from Zone II of chromatogram 1C.

shaker. After × 10 magnification by a photographic enlarger, the sections were measured to the nearest mm. The histograms (Figs. 1 and 2) show the enhancement (+) or retardation (−) of the growth in length of the sections, expressed

as a percentage of the increase in length of the controls (elution from a paper square behind the starting line). The average 5 per cent. fiducial limit calcu-

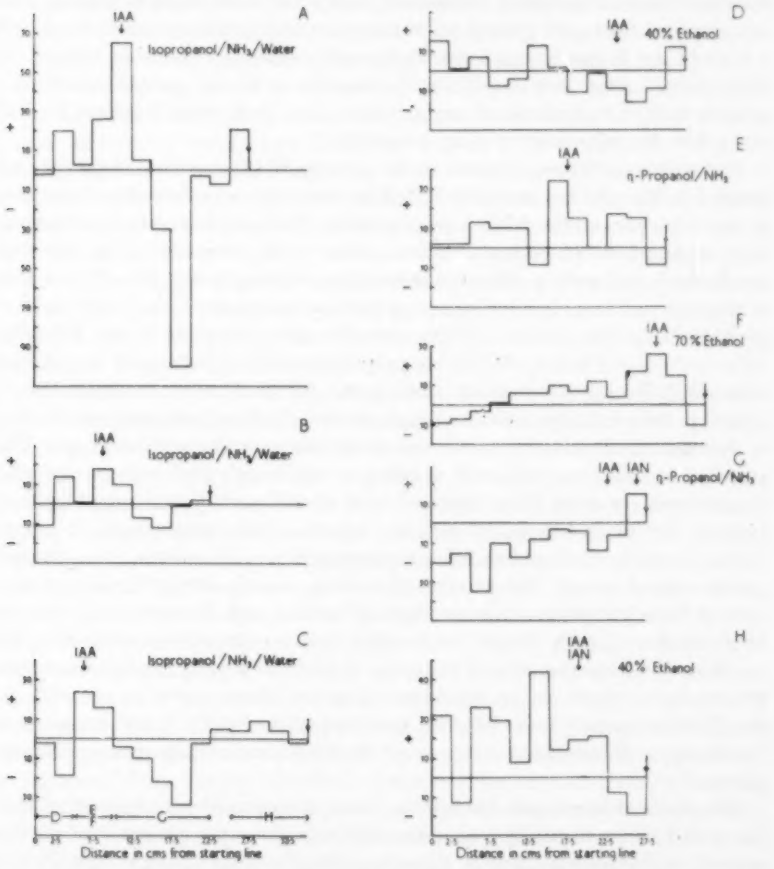

FIG. 2. Chromatograms of the *ethyl acetate fractions* showing growth activity of eluates. Histograms plotted as in Fig. 1. Key: IAA and IAN = position of marker spots.

Fig. 2A. Total fraction = 70g. fresh wt. root.

Fig. 2B. Neutral fraction = 40g. fresh wt. root.

Fig. 2C. Acid fraction = 40g. fresh wt. root.

Fig. 2D–H. Eluates from zones of corresponding letters marked on chromatogram 2C. Each eluate = 70g. fresh wt. root.

lated from the standard errors of the means of control coleoptile sections from all experiments reported was 16 per cent.; growth enhancements or inhibitions greater than this value are taken as significant.

RESULTS

Aqueous fraction. This fraction, obtained from different batches of roots, has been chromatographed, bioassayed, and tested with reagents on a number of occasions. Strongest growth stimulatory activity was always associated with a zone (Zone I) containing a reactor (purple colour) to Ehrlich's reagent. In addition, a rather less well defined zone (Zone II) of growth-stimulatory activity was always obtained running from just in front of a yellow Ehrlich reactor to the solvent front (Fig. 1A and C).

Treatment of chromatograms of the aqueous fraction with acetone-Ehrlich reagent in the cold has always revealed the two reactors referred to above, one giving a purple and the other a yellow colour. The purple reactor corresponds with tryptophane in giving a yellow colour with nitric-nitrite, purple with xanthydrol, pink with *p*-dimethylaminocinnamaldehyde and blue-purple with ninhydrin-pyridine, but no reactions with persulphate, sulphanilic acid or *p*-nitraniline. The reactor has the same Rf as tryptophane in the following solvents: phenol/water, Rf 70; isopropanol/ammonia/water, Rf 21; 20 per cent. KCl, Rf 44; and butanol/acetic/water, Rf 48. Further evidence for the identity of the unknown with tryptophane was obtained following purification of the aqueous fraction by the technique of Asatoor and Dalgliesh (1956). The aqueous fraction was adjusted to pH 4·0 with acetic acid and treated with deactivated charcoal. The charcoal was eluted with 7 per cent. aqueous phenol, the phenol removed and the residue chromatographed. A purple Ehrlich reactor of the same Rf as tryptophane and stimulatory to coleoptile growth was detected. The growth-promoting activity of this Ehrlich reactive spot of Zone I from an aqueous fraction purified with Dowex 50 × 8 has also been compared with that of its L-tryptophane content determined by the method of Undenfriend and Peterson (1957) on a parallel chromatogram. Percentage increase over control growth in the bioassay was 24 per cent. for the Ehrlich reactive zone only (i.e. not the whole of Zone I) and 21 per cent. for its tryptophane equivalent (12 μg.) eluted from a simultaneous chromatogram.

Bioassays of isopropanol/ammonia/water chromatograms of aqueous fractions and of resin-purified aqueous fractions show the presence of further growth-stimulatory activity in Zone I running in front of the purple Ehrlich spot (Fig. 1A). This activity is not due to tryptophane but may result from the high concentration of other amino-acids in this region of the chromatograms.

Booth (1958) has shown that, when sucrose is absent from the bioassay medium, sugars present in plant extracts are capable of stimulating coleoptile straight growth. The following observations, however, strongly point against such sugars being involved in the activities detected on chromatograms by our present technique. The assay of a butanol/acetic/water chromatogram of the aqueous fraction is shown in Fig. 1B. The major peak of growth-stimulatory activity is again correlated with the purple Ehrlich reactor. This solvent effects a good separation of substances reacting with aniline oxalate, ninhydrin, and

Ehrlich reagents and the regions giving positive reactions with aniline oxalate (sugars) are regions of no or very low growth-promoting activity (Fig. 1B). The use of the Dowex resin has enabled us to obtain a sugar fraction (free from ninhydrin-positive material) and a ninhydrin-positive fraction (free from sugars). The former lacked growth-promoting activity, the latter reproduced the growth-promoting activity of the original aqueous fraction.

Zone II on our primary chromatograms developed with isopropanol/ammonia/water corresponds with a ninhydrin-positive streak. However, on elution and rechromatography in the same solvent three defined ninhydrin reacting areas are detected (labelled A, B, and C in Fig. 1E). Zone C which is associated with the only region of possibly significant growth activity gives, on elution and chromatography in butanol/acetic/water, two ninhydrin spots of Rf 60 and 68. Marker spots of amino-acids run on a parallel chromatogram had the following Rfs.: isoleucine 58, leucine 61, phenylalanine 50, valine 45, and methionine 43. However, chromatography of the eluate from Zone C in tertiary butanol/ethylmethylketone/formic acid/water gave a single ninhydrin spot clearly separated from these five amino-acids.

From the behaviour on rechromatography of eluates from chromatograms of their aqueous fractions Britton *et al.* (1956) and Audus and Gunning (1958) have described the presence of interconvertible substances stimulatory to coleoptile growth. Fig. 1C shows a primary chromatogram of our aqueous fraction from which Zones I and II were eluted. On rechromatography in isopropanol/ammonia/water each zone produced only one significant peak of growth-promoting activity at the original position (Fig. 1D and E).

Britton *et al.* (1956) report a yellow Ehrlich reactor present on chromatograms of their aqueous fraction and correlated with activity in the coleoptile test. As earlier indicated, we have detected a region giving an immediate yellow colour with acetone Ehrlich reagent in the cold and having the following Rf values: isopropanol/ammonia/water, Rf 30–44 (Fig. 1A); butanol/acetic/water, Rf 51 (Fig. 1B); 20 per cent. KCl, Rf 61. This region has, however, no growth-promoting activity at the concentrations tested. It could not be separated from urea in the above three solvents or with tertiary butanol/ethylmethylketone/formic acid/water. Urea and ureides give immediate yellow colours with this Ehrlich reagent. When this spot is ringed with phenol red and treated with urease (Bollard, 1959) the indicator turns red indicating liberation of ammonia. These tests show the yellow Ehrlich reactor on our chromatograms to be urea, but it must be borne in mind that this may have arisen from breakdown of ureido-compounds during the period when the extract is at pH 3. The yellow Ehrlich reactor is always overlapped on isopropanol/ammonia/water chromatograms by a substance which is presumably phenolic since it gives an intense red colour with sulphanilic acid and an immediate blue colour with *p*-nitraniline. This compound also does not coincide with growth-promoting activity.

Ethyl acetate fractions. Isopropanol/ammonia/water chromatograms of the total ethyl acetate, neutral, and acid fractions are shown in Fig. 2A, B, C.

Elution and rechromatography of zones from chromatogram Fig. 2C gave the chromatograms 2D to 2H. No colour reactions have been obtained with any of these chromatograms of the ethyl acetate fractions even using 1 per cent. *p*-dimethylaminocinnamaldehyde in 6N.HCl/ethanol (1:1), reported to be a more sensitive test for indoles on paper than the standard Ehrlich reagent (private communication from Dr. J. Harley-Mason, Department of Chemistry, University of Cambridge).

DISCUSSION

Aqueous fraction. Britton *et al.* (1956) reported the absence of any characteristic indole reactions on chromatograms of their aqueous fraction of excised tomato roots. By contrast, chromatograms of our aqueous fraction always carried a well-defined spot reacting purple with Ehrlich reagent. This reactor appears to be identical with tryptophane and the amount present to be capable of causing significant growth stimulation in the coleoptile assay. The total growth-promoting activity of Zone I is not fully accounted for by its tryptophane content and is associated with other and as yet unidentified ninhydrin-positive material.

Bentley (1958) reports that 'alkaline saponification of tryptophane (N.NaOH at 15 lb./in.^{2}) does not yield extractable auxins yet under these conditions highly active indole compounds possibly obtained from indole complexes occur in plant extracts'. In our experience tryptophane on heating (autoclaving at 15 lb./in.^{2} for 5 min. or refluxing for 5–10 hrs.) at neutral or slightly acid pH undergoes considerable activation as judged by assay with the coleoptile straight growth test (Fig. 3) or when tested on the growth of the excised rye root (Roberts and Street, 1955). Isopropanol/ammonia/water chromatograms of refluxed tryptophane show at least three fluorescent zones absent from chromatograms of untreated tryptophane and strong growth-stimulating activity and colour reactions with Ehrlich's (purple) and nitric-nitrite (pink) reagents at the IAA position. On solubility grounds, tryptophane would be expected to be concentrated in the aqueous fraction and not, as seems to be suggested by Bentley (1958), in the ether fraction. The recognition of tryptophane as the major indole constituent of our aqueous fraction suggests that it may be generally present in such aqueous fractions and may readily act as a source of auxins during extraction procedures as well as itself contributing to the growth-stimulatory activity recovered from chromatograms by elution.

Elution of active zones from our aqueous fraction chromatograms has failed to yield positive evidence of their interconvertibility. Thus with isopropanol/ammonia/water our Zone I (equivalent in Rf to Zone X of Britton *et al.*, 1956) and our Zone II (which would include Zones Y and Z of these workers) when eluted and rechromatographed in the same alkaline solvent produced in each case only one zone of significant growth-promoting activity. The possibility should be borne in mind that the conclusion that interconvertible substances are present might follow erroneously from the elution and rechromatography of zones containing massive amounts of several constituents (particularly with

heavily loaded chromatograms); the elution effecting some purification and the rechromatography permitting more effective separations. For instance, the aqueous fraction contains sugars and these would, in our experience, be

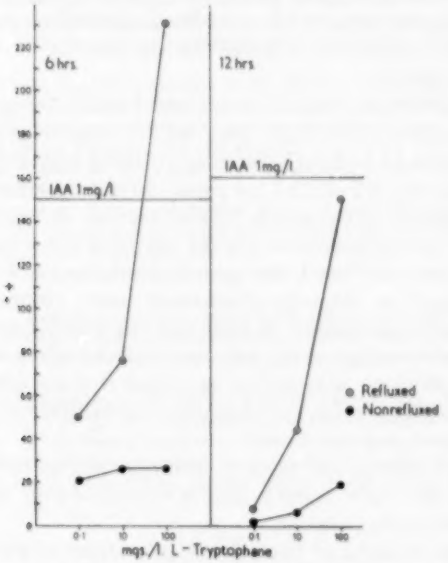

FIG. 3. Activation of L-tryptophane by refluxing a dilute neutral aqueous solution for 10 hrs. Enhancement of growth of *Avena* coleoptile sections expressed as a percentage of the increase in length of control sections. Measurement made after 6 and 12 hrs. treatment. Horizontal line shows the response to 1 mg./l. IAA.

expected, particularly when effectively separated from other constituents, to be recorded as zones of activity when using, as did Britton *et al.* (1956) a bioassay medium devoid of sucrose.

Ethyl acetate fractions. Patterns of growth activity in the ethyl acetate fraction chromatograms resemble, in general, those already reported in the literature: growth stimulation at the IAA position, inhibition at the β-position and stimulation at the solvent front (Bennet-Clark and Kefford, 1953; Lexander, 1953).

Chromatograms of the acidic ester-soluble material have been divided into four zones for rechromatography (Fig. 2C). The first zone, including the starting line when rechromatographed in 40 per cent. ethanol, failed to fractionate into zones of growth-inhibiting or promoting activity (Fig. 2D).

Chromatograms of the total, acid, and neutral ethyl-acetate fractions have all displayed some growth-stimulatory activity at the Rf of IAA. When eluates have been made of this zone from isopropanol/ammonia/water chromatograms of the acidic ester-soluble material and rechromatographed in 70 per cent. ethanol (Fig. 2F) and *n*-propanol/ammonia 60/40 (Fig. 2E), stimulation

has again been observed at the position of IAA. Growth-promoting activity is, however, also observed at the Rf of IAA with ethyl acetate neutral fractions (Fig. 2B), the chromatogram of the neutral fraction being in fact interpretable as a 'dilute' form of its corresponding acid fraction (Fig. 2C). Because of its lower activity, elution of the 'IAA' zone from neutral fraction chromatograms has yielded less conclusive evidence for its identity in chromatographic behaviour with IAA.

The results of Bennet-Clark, Younis, and Esnault (1959), using a starch column for separation of the acidic ester-soluble material of roots, casts doubt on the identity with IAA of the substance(s) causing maximum growth stimulation and having the Rf of IAA on paper chromatograms. The activity of the active fractions from the starch column was lost on chromatography and these workers therefore concluded that the activity was due to volatile material. It should, however, be noted that growth-promoting activity at the Rf of IAA was obtained on isopropanol/ammonia/water chromatograms of the initial ethyl acetate acid fraction of their root extracts and that loss of activity by volatilization was apparently only encountered when dealing with the column eluates. We have obtained no indication that our activity at the Rf of IAA is volatile during evaporation of the ethyl acetate fractions or during concentration of chromatogram eluates.

The inhibitory zone of our extract, corresponding in Rf to inhibitor β, is concentrated in the acidic fraction and is reproduced on rechromatography in *n*-propanol/ammonia (Fig. 2G).

Elution of the material at the solvent front from isopropanol/ammonia/water chromatograms and rechromatography in 40 per cent. ethanol has revealed the presence of growth stimulators not detected on the initial chromatograms. The zones of growth on ethanol chromatograms do not correspond in Rf with IAN (Fig. 2H).

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Effects of Inhibitory Concentrations of 3-Indolylacetic Acid and 3-Indolylacetonitrile on Cell Division and Tissue Differentiation in Excised Tomato Roots

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SUMMARY

Inhibitory concentrations of 3-indolylacetonitrile (IAN) cause, in cultured excised tomato roots, a marked decrease in the rate of cell division at the apical meristem but only a slight reduction in the lengths of mature exodermal and cortical cells. The reduced rate of cell division is associated with a decrease in the number of meristematic cells at the root apex. By contrast, 3-indolylacetic acid (IAA) causes marked reduction in the lengths of mature cortical cells but does not markedly reduce cell-division rate at the apical meristem.

Various lines of evidence indicate that both IAA and IAN cause a relative increase in the number of longitudinal and a decrease in the number of transverse division walls in the meristematic zone of the root apex.

Partial inhibition of the linear growth of excised tomato roots by IAA and IAN is accompanied by increases in root and stelar diameters. These increases result from radial enlargement of the cortical cells and increase in the number of stelar cells in the transverse section. The enlarged steles contain an increased number of lignified xylem elements, but only with the most inhibitory concentration of IAN (10^{-4}g./ml.) is there evidence of the development of secondary xylem. Both auxins increase significantly the xylem vessel unit length.

INTRODUCTION

THE effects of inhibitory concentrations of 3-indolylacetic acid (IAA) and 3-indolylacetonitrile (IAN) on the morphology and linear growth of excised tomato roots have been described in an earlier paper (Street, McGregor, and Sussex, 1954). The present investigation has been devoted to examining the effects of these auxins on root anatomy and cell differentiation.

EXPERIMENTAL METHODS

General experimental procedures were as previously described (Street, McGregor, and Sussex 1954).

Techniques used to study cell-division rates and cell expansion. Exodermal cell lengths were measured 5 mm. from the root apex by the technique previously described (Street *et al.*, 1954). Cell counts were made by a modification (Butcher and Street, 1960) of the maceration technique of Brown and Rickless

(1949). Each value for cell number was calculated from a count of the number of cells in 0·9 mm.^{3} of the suspension on the Haemocytometer slides.

The preparation and examination of transverse and longitudinal sections. Roots collected after 7 days' growth were fixed in formalin-aceto-ethanol, preserved in 70 per cent. ethanol, dehydrated by the butylalcohol technique (Johansen, 1940), and embedded in paraffin wax. Median longitudinal sections were cut at 25 μ and transverse sections at 40 μ thickness, attached to the slides by Haupt's solution, and stained by Sharman's method (Sharman, 1943). Transverse sections were used for determinations of root and stelar diameters, numbers of cells in different tissues and cell layers, and diameters of cortical cells. The values for root and stele diameter from each section were means of two measurements, one in the plane of the single line of primary xylem vessels and the other at right angles to this. Xylem vessels not in the single line of vessels, which constitutes the primary diarch pattern, have been termed 'additional' vessels. The primary line of xylem vessels was used as a dividing line in determining the number of non-lignified cells in half the circumference of layers such as the endodermis. In measuring diameters of cortical cells, 20 cells were measured per section, 5 along each of the 4 radii at right angles. Longitudinal sections were used for measurements of cell dimensions and for determination of the distances from the promeristem at which characteristic features of cell differentiation were recognizable. In determining cell dimensions, up to 10 cells per root were measured either from a single section or using adjacent sections.

By assuming a uniform rate of extension growth during the culture period distances from the promeristem have also been considered in terms of time from initiation at the promeristem. This has enabled estimates to be made as to whether the rates of differentiation of individual tissues are markedly affected by the auxin treatments.

RESULTS

Effects of IAA and IAN on cell expansion and cell division. Previous work (Street *et al.*, 1954) suggested that, at concentrations causing up to 50 per cent. inhibition of linear growth, IAA acts primarily by causing a decrease in length of the expanded cells whereas IAN acts by causing less marked decreases in mature cell lengths accompanied by a decrease in the mean rate of cell division at the root-tip meristem. This view has now been further examined by experiments involving measurements of the lengths of expanded exodermal cells and of the total numbers of cells in root apices. Increments of extension growth of main root axes were determined for the culture periods 24–48 hrs. and 144–168 hrs. and in each case immediately after the second measurement 20 replicate root tips, each equal in length to the mean increase in length during the test 24-hr. period, were excised and bulked to give the cell suspension. Since each root segment includes the root-tip meristem the total cell number per segment does not represent the new cells produced during the test period. A lower number of non-vacuolated cells in the treated

as against the control segments may, however, be regarded as indicative of a reduction in size of the meristem and the number of vacuolated cells per segment will approximate to the number of cells determining the increase in root length.

The results presented in Table I show that inhibitory concentrations of IAN cause a marked decrease in the number of vacuolated and non-vacuolated cells but effect only a slight reduction in exodermal cell lengths. By contrast,

TABLE I

The effects of IAA and IAN on linear growth-rates, average lengths of mature exodermal cells, and numbers of meristematic cells at the root apices of excised tomato roots

Test period	Treatment	Mean length of macerated root segments (mm.) (20 replicates) (A)	Mean number of cells per segment $\times 10^{-4}$		Mean length of exodermal cells (μ) (5 replicates) (C)	$\frac{A}{B \times C} \times 100$
			Non-vacuolated	Vacuolated (B)		
24–48 hrs.	Control	10·2 ± 0·6*	1·94	1·60	93·0 ± 2·6*	6·8
	10^{-5}g./ml. IAA	5·4 ± 0·3	1·62	1·23	3·6 ± 2·2	6·9
	$10^{-4\cdot5}$g./ml. IAA	2·3 ± 0·2	1·50	1·13	51·0 ± 2·3	4·8
	Control	9·9 ± 0·7	2·0	1·77	93·6 ± 8·4	6·0
	10^{-5}g./ml. IAN	3·0 ± 0·3	1·01	0·73	84·6 ± 4·1	4·8
	$10^{-4\cdot5}$g./ml. IAN	2·1 ± 0·3	0·80	0·55	80·4 ± 3·8	4·8
144–168 hrs.	Control	13·5 ± 0·5	2·46	2·39	89·4 ± 3·0	6·3
	10^{-5}g./ml. IAA	10·4 ± 0·3	2·44	1·79	73·6 ± 4·4	8·0
	$10^{-4\cdot5}$g./ml. IAA	3·1 ± 0·2	2·12	1·15	53·4 ± 1·8	5·0
	Control	11·7 ± 0·7	2·26	2·0	94·2 ± 3·2	6·2
	10^{-5}g./ml. IAN	6·4 ± 0·3	1·35	1·33	88·8 ± 3·2	5·4
	$10^{-4\cdot5}$g./ml. IAN	4·4 ± 0·4	1·27	0·98	80·4 ± 5·1	5·6

* Standard errors.

IAA causes a more marked reduction in exodermal cell length and a relatively less-marked fall in cell numbers. IAA and IAN are particularly contrasted in that the one does not and the other does markedly decrease the number of non-vacuolated cells. Division of the increment of linear growth (A) by the product of the number of vacuolated cells (B) and the exodermal cell length (C) gives for all treatments values of the same order as for the control roots, suggesting that B and C are valid estimates of the two primary factors determining linear growth. The decreases in the value of this fraction by both IAN concentrations and by the higher IAA concentration may result from an effect of the auxins on the orientation of cell division walls. This view is supported by the increases in the number of cells per transverse section revealed by the anatomical investigation now to be described.

Effects of IAA and IAN on root diameter and on the number of cells constituting the transverse section. Sections of roots cultured in presence of IAA at 10^{-7}, 10^{-8}, and 10^{-9} g./ml. and in presence of IAN at 10^{-4}, 10^{-5}, and 10^{-6} g./ml. were examined and compared with those from roots cultured simultaneously in standard medium. Serial sections were prepared for an initial distance of 1,000μ and measurements made on sections corresponding to 100, 300, 500,

and 1,000 μ from the promeristem. Sections were also examined at 1·5, 2·5, 5, 10, 15, 20, 25 mm. from the promeristem (except at the most inhibitory auxin concentrations where extension growth during the culture period was less than 25 mm.).

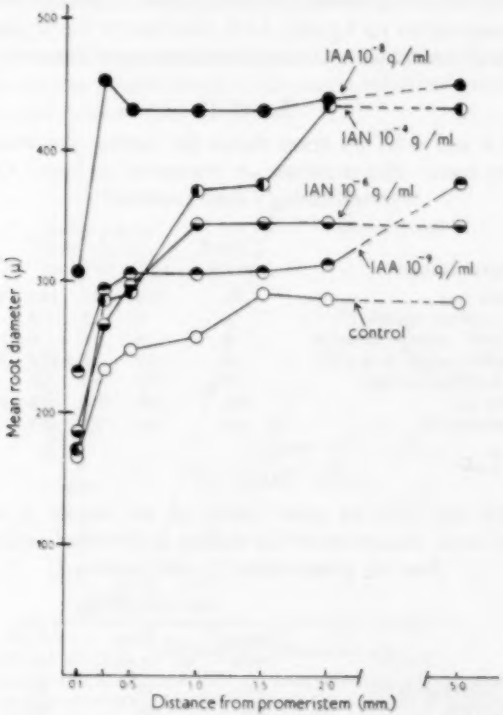

FIG. 1. The influence of IAA and IAN on mean root diameter of excised tomato roots cultured for 7 days in presence of these auxins.

The total data has been reviewed in support of the conclusions presented although only representative values are quoted in the tables and figures. Where attention is drawn to differences in anatomical values resulting from auxin treatment these have been examined statistically using the whole data and shown to be significant at the 1 per cent. level.

Increases in root diameter resulting from auxin treatment are shown in Fig. 1. These increases involve increases in stele diameter and also, with IAA at 10^{-7} and 10^{-8} and with IAN at 10^{-4} g./ml., increases in cortex thickness (Table II). The increases in stele diameters are reflected in the increased numbers of primary xylem vessels; the increases in root diameters are reflected in the numbers of cells in half the circumference of the piliferous layer. Enhanced cortex thickness is roughly paralleled by increased values for

cortex cell diameter and observation confirms that the auxins do not effect significant changes in the number of layers of cells in the cortex (Table II).

There are significant increases in the number of 'additional' xylem elements and in the number of non-lignified stelar cells in response to both IAA and IAN (Table II). The very marked increase in the number of additional xylem elements in response to 10^{-4} g./ml. IAN was due to the appearance of concentric groups of vessels at distances greater than 2 mm. from the promeristem.

TABLE II

Effects of IAA and IAN on mean values for various anatomical features of excised tomato roots. Measurements on transverse sections, 5 mm. from the promeristem (3 roots examined)

Character measured	Contol roots	+IAA g./ml.			+IAN g./ml.		
		10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Diameter of stele (μ)	82	124	123	162	124	111	111
No. of primary xylem vessels	4	6	7	9	8	8	8
No. of 'additional' xylem elements	2	2	7	4	1	4	19
No. of non-lignified cells in ½ stele	47	103	97	132	107	96	59
No. of cells in ½ piliferous layer	37	62	45	50	76	66	50
Cortex thickness (μ)	102	128	164	132	113	114	162
Cortex cell diameter (μ)	22	24	33	29	24	25	31

TABLE III

Effects of IAA and IAN on mean values for the lengths of certain cells in excised tomato roots. Measurements on median longitudinal sections at 2·5 mm. from the promeristem (3 roots examined)

Tissue	Cell lengths (μ)		
	Control roots	+IAA 10^{-7} g./ml.	+IAN 10^{-5} g./ml.
Piliferous layer	22	29	50
Cortex	83	30	60
Endodermis	46	21	36

It was only with this high IAN concentration that there was any clear indication of a 'secondary' origin of some of the xylem vessels. Despite the enhanced numbers of cells in the stele of roots inhibited in linear growth by the auxins neither a continuous cambium nor localized cambia were observed.

Effects of IAA and IAN on cell lengths and diameters as measured from median longitudinal sections. Both IAA and IAN enhanced the length of piliferous layer cells (Table III). This, combined with the increase in the number of cells in the circumference of this layer, points to an increase in radial longitudinal and a decrease in transverse division walls in this layer as a result of auxin treatment. Endodermal cell lengths followed values for other cortical cells in that they were more markedly reduced during IAA than during IAN inhibition of root growth (Table III). The parenchymatous cells of the stele were either unaffected or, at high auxin concentrations, reduced in both

length and radial diameter by both IAA and IAN. The diameter of the xylem vessels was relatively unaffected by the auxins, but with concentrations causing only a moderate inhibition of linear growth (10^{-9} IAA and 10^{-5} g./ml. IAN) a very significant enhancement of xylem vessel unit length occurred (Fig. 2). These concentrations did not significantly alter the mature lengths of the sieve tube units.

Although xylem differentiation and the differentiation of other cell types occurred nearer to the promeristem in the auxin inhibited roots, this did not involve more rapid rates of differentiation.

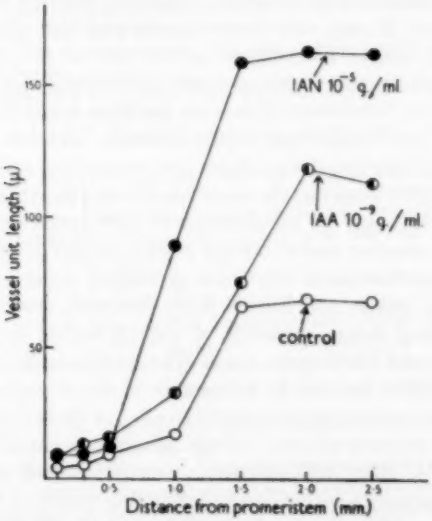

FIG. 2. The influence of IAA and IAN on mean values for xylem vessel unit length in excised tomato roots cultured for 7 days in presence of these auxins.

DISCUSSION

The contrast between IAA and IAN in their relative effects on cell division and cell expansion may be explained in a number of ways. It may be that these two auxins act independently. Alternatively, if IAN must be converted to IAA in order to show auxin activity, as has been suggested by Stowe and Thimann (1954), then either the distribution of absorbed IAN within the root must be strongly contrasted with that of IAA or, during cell maturation, marked changes must occur in the activity of the conversion system. The studies of Seeley, Fawcett, Wain, and Wightman (1956) show that the tissues of a number of species, including tomato, produce small quantities of indolylcarboxylic acid from IAN but no or only traces of IAA.

The inhibition of cell division at the main-tip meristem resulting from high IAN concentration is correlated with an enhanced number of cells in the transverse section of the stele and with the development of secondary xylem.

These findings are in agreement with the earlier observation (Street *et al.*, 1954) that when roots are inhibited by IAN there are, in regions devoid of laterals and often also in the regions between closely set laterals, generalized cell divisions in the pericycle. The failure of excised tomato roots to develop organized secondary vascular tissues and the occurrence of, at best, disorganized cambial regions in the steles of old cultures (Dormer and Street, 1948) indicate that the normal stimulus to secondary growth is absent in these root cultures. At appropriate concentrations externally applied IAN causes a significant increase in the frequency of lateral root initials (less cells separating points of lateral initiation) (Street *et al.*, 1954) and induces early development of secondary xylem. It may, therefore, be suggested that either IAN is or can substitute for the normal stimulus to cell division in the stelar parenchyma which promotes these two aspects of root development in the intact plant. Recent data from our laboratory (Thurman and Street, 1960) strongly supports the view that excised tomato-root cultures contain IAA but chromatographic analysis has so far failed to demonstrate the presence of an auxin agreeing in properties with IAN. This may be contrasted with the evidence presented by Nitsch and Nitsch (1955) for the presence of IAN in the intact tomato plant.

Increasing the sucrose concentration of the culture medium above 2 per cent. results in a reduction of the linear growth of excised tomato roots and the roots become yellow and brittle. Root diameter, cortex width, and the number of lignified xylem elements in the transverse section are also all increased (Street and McGregor, 1952). The high sucrose concentrations do not significantly affect the rate of maturation of the xylem vessel units or the duration of the expansion phase of cortical cells. In these features roots grown in media of high sucrose content closely resemble roots inhibited in growth by IAA or IAN. Whether high sucrose concentration leads to enhanced levels of endogenous auxins or whether some of the major effects of externally applied IAA and IAN are to be explained in terms of their effects on carbohydrate uptake and metabolism is, however, not yet determined.

Pfeffer (1893) and Kojima (1931) showed that a mechanical inhibition of root growth causes the differentiation of xylem cells to approach the meristem but that when this is considered in terms of the age of cells then differentiation is seen to be delayed rather than speeded up. Torrey (1953) has, however, reported that IAA increases the *rate* of xylem maturation. This effect was, however, observed during the first 24 hours following transfer from a medium devoid of, to a medium containing, an inhibitory concentration of IAA. Following the longer auxin treatments used in this investigation, the promotion of lignification by IAA reveals itself in the increased number of xylem vessels and in the increased length of the vessel units. These increases in the number of xylem vessels suggest that both IAA and IAN increase the incidence of longitudinal divisions in the procambial strands and that there is a corresponding decrease in transverse divisions compensated for by the enhanced elongation of the vessel-unit mother cells. Apparently, a similar reorientation of division walls occurs in the piliferous layer. Evidence for a reorientation of division

walls has also been obtained in studies of the inhibition of excised root growth by α-naphthaleneacetic acid (Butcher and Street, 1960). These observations are in accord with earlier work by Levan (1939) and Burström (1942) and support Åberg's view (1957) that a change in the polarity of the root cells is involved in the inhibition by auxins of the extension growth of roots.

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The Effects of Gibberellins on the Growth of Excised Tomato Roots

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SUMMARY

1. At appropriate concentrations both gibberellic acid (GA) and 1-naphthaleneacetic acid (NAA) enhance the main axis growth of excised tomato roots grown in culture media containing sucrose at concentrations below 1 per cent. Lateral root extension growth is enhanced by GA at all sucrose concentrations tested; only at the lower sucrose concentrations is this effect observed with NAA. Both GA and NAA increase the number of emergent lateral roots and this effect is most marked in media of low sucrose content. Both GA and NAA at higher concentrations inhibit root growth but NAA exhibits its full range of growth effects over a much narrower concentration range than GA.

2. GA, like NAA, speeds up the loss of meristematic activity which occurs when individual meristems are repeatedly subcultured in media containing 1 per cent. or higher concentrations of sucrose.

3. The promotion of main axis growth by both GA and NAA involves enhanced cell elongation and cell division. At a moderately inhibitory concentration GA reduces both cell elongation and cell division; this is not the case with NAA.

4. Gibberellins A_1, A_2, and A_4 resemble GA (gibberellin A_3) in their growth effects. *Allo*gibberic acid like GA promotes lateral root extension growth but causes marked inhibition of root growth at a much lower concentration than GA.

INTRODUCTION

THE root growth of intact plants of a number of species is unaffected or inhibited by gibberellic acid added to the culture solution (Brian, 1959). Lee (1959), using excised root clones derived from a tall and a dwarf variety of tomato, reported inhibition of main axis growth in response to gibberellic acid. Richardson (1958) working with seedlings of *Pseudotsuga menziesii* and Whaley and Kephart (1957) using excised radicle tips of certain strains of maize have, however, briefly reported that gibberellic acid can stimulate the extension growth of the root.

Brian (1959) has reviewed the overlap in the activity spectra of gibberellic acid and auxins. Previous work has shown that the responses of excised root cultures to auxins is critically affected by their physiological age and nutritional status (Street, 1957). The present paper describes a study of the influences of the sucrose concentration of the medium and the age of the root meristem on the growth responses of excised tomato root cultures to gibberellins and to the auxin, 1-naphthaleneacetic acid. These experiments have enabled us to define conditions under which gibberellic acid will respectively

stimulate or inhibit root growth and to expose differences between the effects of gibberellic acid and of an auxin.

METHODS

The general culture techniques have been previously described (Hannay and Street, 1954; Ferguson, Street, and David, 1958). Experimental cultures were initiated from 10 mm. tomato root tips (clone of Best-of-All) excised from 6-day-old sector cultures in 1·5 per cent. sucrose medium. The incubation conditions and the recording of linear root growth were as previously described (Street, McGregor, and Sussex, 1954). The values for root dry weight were determined by drying for 12 hours at 98° C. In certain experiments, the cultures were carried through a sequence of incubation periods of 7-days (Passages) by each time excising the main axis tips (10 mm.) and transferring these to culture flasks of fresh medium.

Cell counts were carried out by the method of Brown and Rickless (1949), modified by centrifuging the cell suspension, removing the supernatant chromic acid, and replacing this with 50 per cent. glycerol. Each cell suspension was obtained from 20 replicate root segments (5–20 mm.) macerated for 16 hours in 2 ml. 5 per cent. chromic acid. The mean value for the cell density was based upon three haemocytometer counts each of not less than 200 cells.

Values for root diameter and for the length and tangential width of exodermal cells at 5 mm. from the apex of the root were determined as previously described (Street and McGregor, 1952).

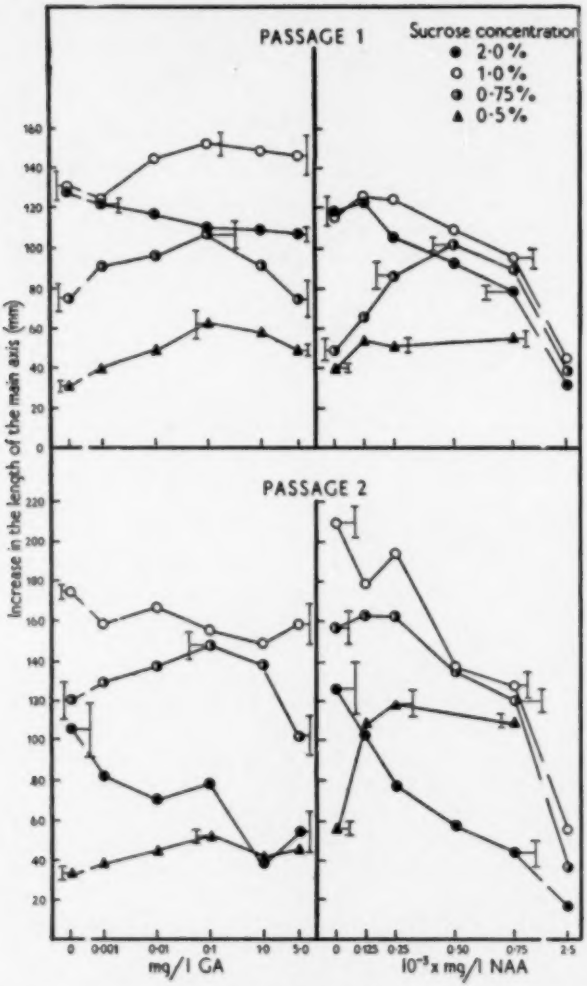
The gibberellins and *allo*gibberic acid were incorporated into the medium by aseptic addition after ethyl ether treatment (Ferguson, Street, and David, 1958). The main sample of gibberellic acid (GA) and the pure sample of *allo*gibberic acid (Brian, Grove, Hemming, Mulholland, and Radley, 1958) were kindly donated by Dr. P. W. Brian, F.R.S., of the Akers Research Laboratory of Imperial Chemical Industries Ltd., and the samples of the gibberellins A_1 ($C_{19}H_{24}O_6$), A_2 ($C_{19}H_{26}O_6$), A_3 (gibberellic acid $C_{19}H_{22}O_6$) and A_4 by Professor Y. Sumiki of the Department of Agricultural Chemistry, the University of Tokyo.

RESULTS

The effects of GA and NAA on the growth of roots in media containing different concentrations of sucrose.

Certain auxins (NAA and 2-naphthoxyacetic acid) reduce excised tomato root growth in culture media of high sucrose content (2 per cent. or higher) but enhance the growth in presence of lower sucrose concentrations (0·75 and 0·5 per cent.). In contrast, certain anti-auxins (α-(1-naphthylmethylsulphide)-propionic acid and 1-naphthoxyacetic acid) decrease root growth in media of low and enhance it in media of high sucrose content (Street, 1955).

The relationship of GA action to auxin action revealed by other workers, therefore, suggested that the sucrose concentration of the culture medium



FIGS. 1–3. The effects of gibberellic acid (GA) and 1-naphthaleneacetic acid (NAA) on the growth of excised tomato roots grown for two successive passages in different concentrations of sucrose.
The vertical lines represent twice the standard errors of the mean values.

FIG. 1. Increase in main axis length (mm.).

might be expected to determine the responses of excised roots to GA. Figs. 1, 2, 3, and 6 show how far alteration of the sucrose concentration of the medium within the range 0·5 to 2·0 per cent. affects the growth responses of excised tomato roots to GA and NAA.

Both GA and NAA reduce main axis growth at high and enhance it at low sucrose concentrations. The upper limit of sucrose concentration compatible with growth stimulation in response to GA and NAA falls on subculture

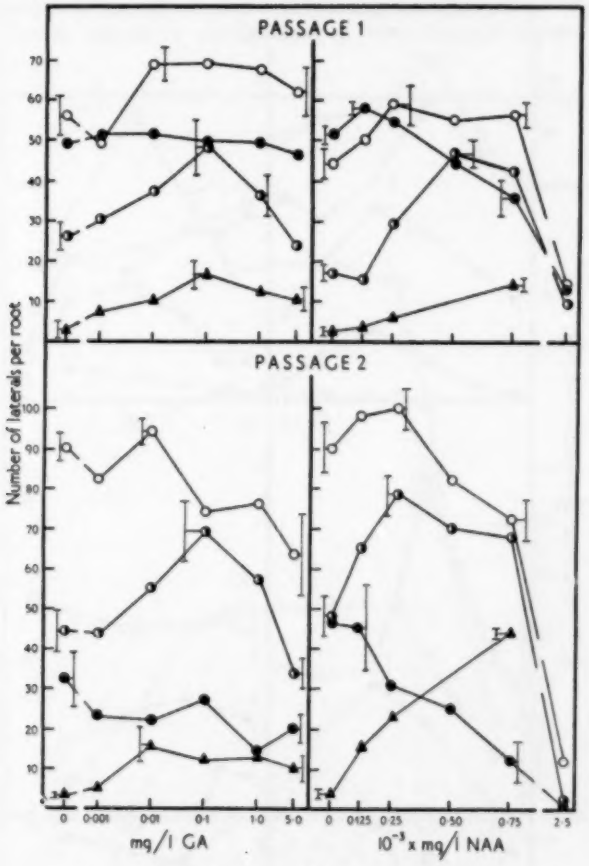


FIG. 2. Number of laterals per root.
(Key as Fig. 1.)

(compare the Passage 1 and 2 responses). Supra-optimal concentrations of the added growth substance are more inhibitory with NAA than with GA; GA only becomes markedly inhibitory at concentrations above 20 mg./l. (Fig. 4) whereas NAA strongly inhibits root growth at 0·0025 mg./l. The concentration range of NAA causing growth stimulation is much narrower than that of GA. The very narrow concentration range over which auxins like NAA significantly enhance root growth has probably determined the view that externally applied auxins only cause inhibition of root growth.

GA and NAA enhance the number of laterals per root, particularly at the lower sucrose concentrations. GA stimulates lateral root extension growth to a greater extent and over a wider range of sucrose concentration than does

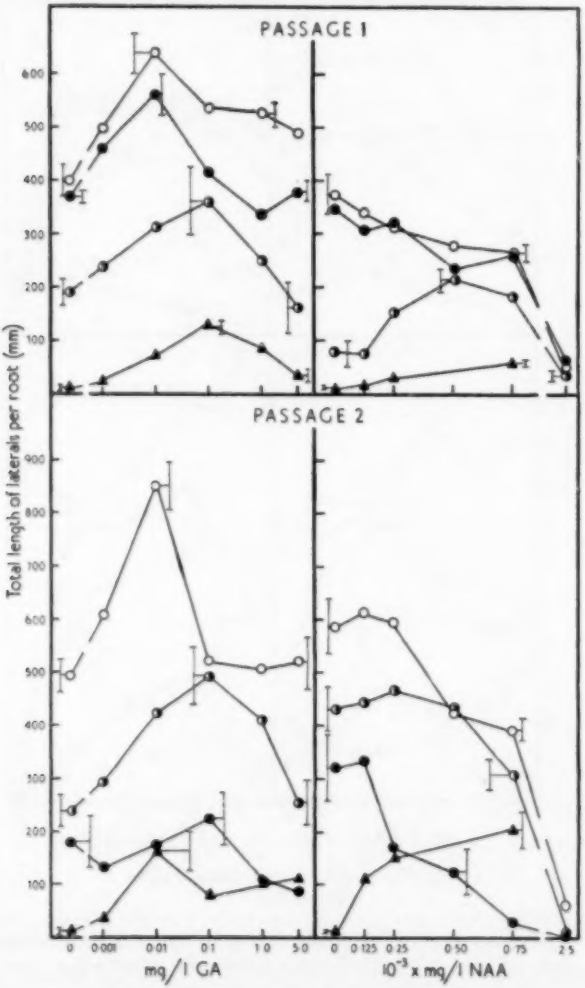


FIG. 3. Total length of laterals per root (mm.).
(Key as Fig. 1.)

NAA. With 1 and 2 per cent. sucrose in Passage 1 and with 1 per cent. sucrose in Passage 2, NAA at all concentrations inhibits whereas GA, at appropriate concentrations, stimulates lateral root extension growth.

The enhancements of growth which occur when roots receive the appropriate concentrations of GA and NAA in media of low sucrose concentration result in significant increases in fresh and dry weight (Table I). For a given enhancement of linear growth, NAA causes a greater increase in fresh and dry weight. This is related to the increase in root diameter caused by NAA (Table II).

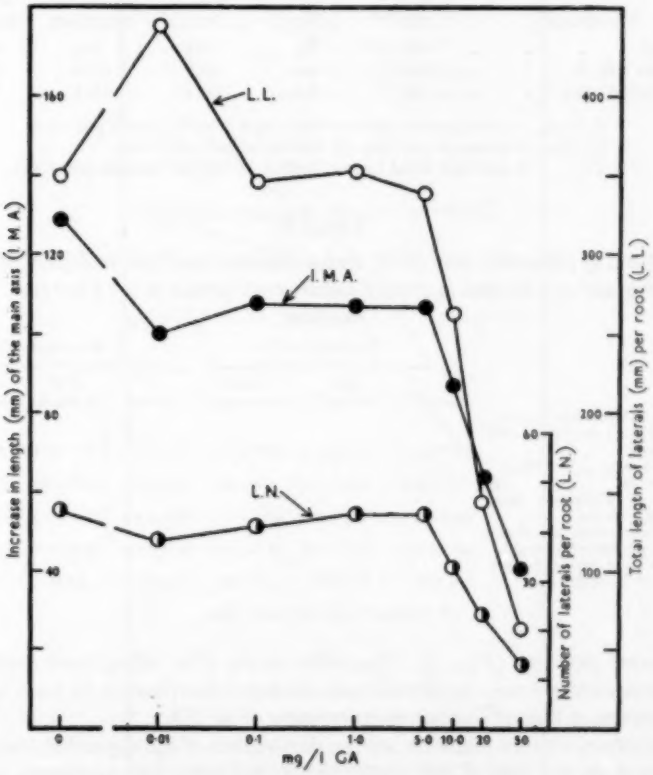


FIG. 4. The effects of supra-optimal concentrations of gibberellic acid (GA) on the growth of excised tomato roots in 2 per cent. sucrose media.

When excised tomato roots are repeatedly subcultured by excision of the main axis meristems they undergo a change which has been referred to as 'ageing' and which leads ultimately to irreversible loss of the meristematic activity of the main root tip (Street, 1957). This 'ageing' process is speeded up by increasing the sucrose concentration of the medium (Street, McGonagle, and Roberts, 1953) or by addition of NAA (Street, 1955). GA similarly enhances 'ageing' in media containing 1 and 2 per cent. sucrose as illustrated by plotting the number of roots growing (the survival number) in each of the

TABLE I

The effects of gibberellic acid (GA) and 1-naphthaleneacetic acid (NAA) on the linear growth, and the fresh and dry weights of excised tomato roots after 10 days incubation in 0·75 per cent. sucrose medium

Treatment	Mean growth values				
	I.M.A.* (mm.)	L.No.†	L.L.‡ (mm.)	Fresh Wt. mg./root	Dry Wt. mg./root
Control . . .	82	19	125	9·3	0·53
GA 0·01 mg./l. . .	122	42	458	20·0	1·04
NAA 0·0005 mg./l. . .	97	54	271	19·5	1·12

* I.M.A. = average increase in main axis length (mm.) per root.
† L.No. = average number of visible laterals per root.
‡ L.L. = average total length (mm.) of visible laterals per root.

TABLE II

The effects of gibberellic acid (GA) and 1-naphthaleneacetic acid (NAA) on cell extension and cell division in excised tomato roots grown in 0·75 per cent. sucrose medium

	Experiment A			Experiment B		
	Control	GA 0·1 mg./l.	NAA 0·0005 mg./l.	Control	GA 10 mg./l.	NAA 0·001 mg./l.
Increase in main axis length (mm.) in 24 hr. (Mean of 20 roots) . . .	6·68 ± 0·5*	15·6 ± 1·2	23·7 ± 1·2	7·8 ± 0·9	6·1 ± 0·8	6·2 ± 0·6
Root diameter (μ). (Mean of 6 roots) . . .	196 ± 8·7	184 ± 5·0	329 ± 23	274 ± 7·7	186 ± 13	374 ± 41
Length of elongated exodermal cells (μ) . .	64·5 ± 2·0	109·6 ± 2·9	98·2 ± 2·7	88·5 ± 3·3	73·5 ± 2·4	97·7 ± 2·9
Tangential width of elongated exodermal cells (μ).	20·7 ± 0·6	22·1 ± 0·6	28·6 ± 0·6	24·0 ± 0·5	19·6 ± 2·1	27·5 ± 0·5
Increase in number of cells per root in 24 hr. . .	6,710	20,870	30,190	7,550	4,170	12,630

* Standard error of mean value.

successive passages (Fig. 5). The roots at the time when their main axis meristems became non-functional were similar in morphology to roots 'ageing' in presence of higher sucrose concentration or of NAA.

The effects on root diameter, on the dimensions of the expanded exodermal cells, and on the rate of cell production at the main axis meristem of both growth-stimulatory and growth-inhibitory concentrations of GA and NAA have been made using roots harvested at the end of the 5th day of incubation. In order to determine the cell production per meristem during the 24 hours before harvesting the following procedure was adopted: at the end of the 4th day measurements were made of the main axis length of 20 roots from each treatment; the roots were then immediately returned to the culture flasks and incubated for 24 hours. The roots were then harvested and again measured. A length of main axis was then cut from each root equal to its extension growth during the 24-hour period and starting 2 mm. from the apex. Such segments will contain the number of cells produced provided the

excluded meristem is not altering in size during the test period. These 20 segments were used to prepare 2 ml. of a cell suspension as previously described and from the haemocytometer counts the cell production in 24 hours per meristem was calculated.

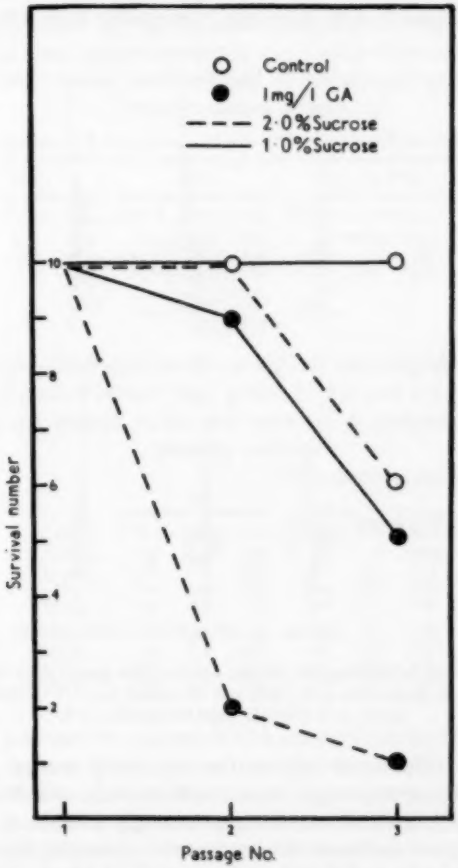


FIG. 5. The effects of gibberellic acid (GA) at 1 mg./l. on the survival of excised tomato roots subcultured by excision of their main axis meristems.

The results obtained (Table II) show that both GA and NAA in enhancing growth in 0·75 per cent. sucrose medium (Experiment A) increase both the rate of cell division and the length of the exodermal cells; only NAA increases root diameter and the width of the exodermal cells. Feucht and Watson (1958) and Greulach and Haesloop (1958) have similarly reported that increased *stem* elongation in *Phaseolus* resulting from GA treatment involves increases in both

cell lengths and rates of cell division. An inhibitory concentration of GA (Experiment B) decreased root diameter, exodermal cell length, and the rate of cell division. A similar degree of linear growth inhibition caused by NAA was associated with an increase in the rate of cell division and in the exodermal cell dimensions. The reduction in linear growth was, however, accompanied by a marked increase in root diameter presumably associated with an aug-

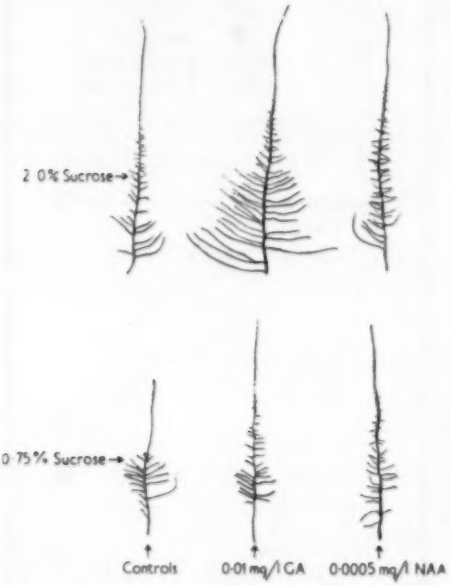


FIG. 6. The morphology of excised tomato roots grown in presence of 0·01 mg./l. gibberellic acid (GA) and of 0·0005 mg./l. 1-naphthaleneacetic acid (NAA). (Shadowgraphs.) × ½.

mented number of rows of cells in the transverse section. Other workers (Levan, 1939; Burström, 1942) have noted that the number of mitoses in roots treated with auxin concentrations strongly inhibitory to their longitudinal growth is not decreased during a period of several days, but that more of the mitoses became orientated in the transverse plane of the root. Åberg (1957) has suggested that this change in the polarity of the cells may be a basic phenomenon in the auxin inhibition of longitudinal root growth.

The effects of gibberellins and of allo*gibberic acid on the growth of excised tomato roots.*

The gibberellins A_1, A_2, and A_4 have been found to resemble very closely gibberellin A_3 (= GA) in their activity. They stimulate lateral root extension in 2 per cent. sucrose medium and also main axis growth and lateral initiation

in presence of lower concentrations of sucrose (Table III). Bukovac and Wittwer (1958), using several bio-assays, have also concluded that the gibberellins all have qualitatively similar growth effects.

TABLE III

The effects of gibberellins A_1, A_2, A_3, and A_4 (each at 0·1 mg./l.), on the growth of excised tomato roots cultured in 0·75, 1·0, and 2·0 per cent. sucrose media. The average growth values are expressed as percentages of the corresponding average control growth

	Growth values in the presence of gibberellins (0·1 mg./l.) as percentages of control											
	GA_1			GA_2			GA_3 (= GA)			GA_4		
Percentage sucrose	I.M.A.	L.No.	L.L.	I.M.A.	L.No.	L.L.	I.M.A.	L.No.	L.L.	I.M.A.	L.No.	L.L.
2·0	84	103	177	84	103	178	79	84	130	84	94	153
1·0	113	136	155	117	151	184	106	143	183	96	116	150
0·75	135	162	221	140	162	182	135	110	189	139	140	156

TABLE IV

A comparison of the effects of gibberellic acid (GA) and allo*gibberic acid (allo-*gA) *on the growth of excised tomato roots grown in 2·0 and 0·75 per cent. sucrose media. The average growth values are expressed as percentages of the corresponding controls*

			Concentration in mg./l.								
			0·01			0·1			5·0		
Passage	Sucrose per cent.	Acid	I.M.A.	L.No.	L.L.	I.M.A.	L.No.	L.L.	I.M.A.	L.No.	L.L.
1	2·0	GA	91	103	152	86	98	109	87	95	103
		allo-gA	98	111	162	90	113	174	35	14	6
	0·75	GA	129	144	162	144	186	185	99	93	83
		allo-gA	103	108	97	124	114	112	63	7	4
2	2·0	GA	68	67	96	75	84	126	51	62	51
		allo-gA	94	112	126	86	104	124	49	14	84
	0·75	GA	123	139	227	123	156	230	85	77	119
		allo-gA	80	77	109	60	19	24	34	0	0

*Allo*gibberic acid was in contrast to GA strongly inhibitory to root growth at 5·0 mg./l. in both 0·75 and 2·0 per cent. sucrose (Table IV). Lower concentrations did, however, like GA, stimulate lateral root extension growth of roots cultured in 2 per cent. sucrose medium. Inhibition of cress seedling root growth by *allo*gibberic acid at concentrations of 10·0 mg./l. or higher has been previously reported by Brian *et al.* (1958). The biological activity of this substance and of other compounds (such as gibberellinic acid and pseudogibberellic acid) which readily arise from GA (e.g. during autoclaving in aqueous solution) requires further detailed study (Brian—private communication).

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Changes in a Leaf-growth Substance in Cotyledons and Primary Leaves during the Growth of Dwarf Bean Seedlings

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SUMMARY

The growth of etiolated dwarf bean leaf disks in darkness was used to assay leaf-growth substances extracted from plant tissues and separated on chromatograms.

A leaf-growth substance, with the same Rf value in isopropanol/ammonia as gibberellic acid, was found in the acidic fraction of extracts from dwarf bean primary leaves and cotyledons. Neither neutral nor basic leaf-growth substances were found in primary leaf extracts.

The amount of leaf-growth substance per cotyledon reached a maximum at the 4th day in cotyledons, whether bean plants were grown in the light or dark. Primary leaves of dwarf bean plants grown in the light reached their maximum content of leaf-growth substance later than the cotyledons; the time of maximum content coincided with the maximum growth-rate of the primary leaves. Leaf-growth substances did not accumulate in primary leaves of plants grown in the dark.

INTRODUCTION

THE dependence of leaf growth on plant-growth substances can be studied by examining the effect of known plant growth-promoting substances on leaves or by extracting substances that influence leaf expansion from growing leaves.

Indole-3-acetic acid (IAA) and other auxins promote the growth of stems and coleoptiles but have little effect on the growth of the leaf lamina, although extension of the veins by IAA distorts leaves. Two new groups of substances that affect leaf growth, the kinins and gibberellins, have been identified. Humphries (1958*a*, *b*) reported that growth of dwarf bean and potato leaves is increased by gibberellic acid (GA) and inhibited by kinetin (Kn). Leaf growth promotion by GA was also reported by Brian, Hemming, and Lowe (1959), Clor, Currier, and Stocking (1958), and Gray (1957). Miller (1956) and Scott and Liverman (1956) reported that Kn promotes growth in etiolated dwarf bean leaf disks.

Gibberellin-like substances were found in dwarf bean seed by Radley (1958) and West and Phinney (1956). Macmillan and Suter (1958) identified the gibberellin from immature runner bean seeds as gibberellin A_1. West and Phinney (1959) isolated two growth factors from immature dwarf bean seed; one was identified as gibberellin A_1 and the other had properties different from

those characteristic of gibberellins. Gibberellins have not hitherto been shown to be present in leaves. Kinetin, originally described as a decomposition product of deoxyribonucleic acid (Miller, *et al.*, 1955), has not been reported as occurring naturally in plant tissues.

The work here described was done to characterize any gibberellins and kinins extracted from dwarf bean primary leaves and cotyledons and to see whether their concentration was correlated with the rate the primary leaves of plants expand when grown in a controlled environment.

MATERIAL AND METHODS

Growth of dwarf bean plants in a controlled environment. Experimental plants were grown under 40 watt 'cool-white' fluorescent tubes in a cabinet kept in a well-ventilated cellar maintained at 25° C. The light intensity, measured by a selenium photo-cell, was 750 f.c. 10 cm. below the centre of the tubes. Under fluorescent light alone, the plants were stunted, presumably because there was too little red and infra-red radiation, but this was overcome by placing incandescent ballast lamps above the tubes. Plants were normally grown in a 16-hour light period and samples were removed at the end of a light period. Bean seeds were graded by weight and planted in John Innes potting compost; the pots were watered daily to constant weight.

Wheat coleoptile section test and etiolated pea epicotyl section test. 10-mm. sections were cut, in red light, from 3-day-old Cappelle Desprez wheat coleoptiles and from the first internode of 4-day-old Meteor pea seedlings grown in darkness at 25° C.

Etiolated dwarf bean leaf-disk test. Selected Canadian Wonder dwarf French bean (*Phaseolus vulgaris*) seedlings were grown for 6 days in moist sand in enclosed tanks at 25°C. in darkness, and 6 mm. diameter disks were cut from the primary leaves, i.e. first pair of opposite leaves above cotyledons, in filtered green light, consisting mainly of radiation within the range 500–570 mμ. The primary leaves were still folded within one another, so four disks could be cut at one time with a cork borer from near the base of each half-leaf. Exposure to dim red light, either from a filtered incandescent lamp or from a filtered red fluorescent tube, promoted the growth of the leaf disks to a significantly greater extent than did the green light. The 15 minutes' exposure to green light while cutting the disks significantly ($P < 0{\cdot}05$) increased growth above that of disks cut in darkness.

The seeds were graded by weight because the sensitivity of leaf disks to growth promotion by GA was greater the larger the seeds from which the leaves were grown. The sensitivity of the disks to Kn appeared to be independent of size of seed. The interaction of seed size and GA effects was significant at the 0·1 per cent. probability level but the interaction of seed size and Kn effects was not significant at the 5 per cent. level. Disks were floated with their adaxial surface in contact with the test solution as the growth-promoting effect of GA and light was greater that way than when the abaxial surfaces were in contact with the solution. The interactions of light and GA with the

orientation of the disks were both highly significant ($P < 0.01$). GA was less effective when the solution contained no sucrose.

Disks were usually cut from the primary leaves of 6-day-old plants. After 5 days' growth, the leaves were too small to give a 6 mm. diameter disk from each half of the leaf; disks from 7- and 8-day-old plants expanded less in 1 per cent. sucrose than those cut from 6-day-old plants. The additional growth produced by GA and Kn was about the same in disks cut from each age of leaf and the interactions with time were not significant. Fig. 1 is the calibration

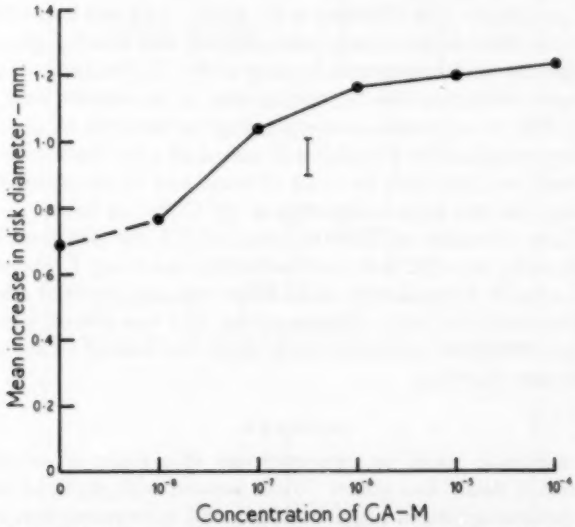

FIG. 1. Calibration of the etiolated dwarf bean leaf disk test for the estimation of GA. Vertical line is three times standard error of mean. Disks were 6 mm. diameter when cut.

curve for estimating GA by the etiolated dwarf bean leaf disk test, using a sample of GA supplied by Plant Protection Ltd.

Extraction of plant material and purification of extracts. Samples of plant material were weighed, and killed by immersion in boiling distilled water for 1 minute. When cool, the material was macerated in the water in which it had been boiled. For extracting acidic substances, dilute phosphoric acid was added to reduce the pH to 3·0 and the material was then macerated with an equal volume of ethyl acetate. After cooling, the macerate was centrifuged and the ethyl acetate removed. The macerate was extracted twice again with volumes of ethyl acetate equal to that removed. The acidic substances in the ethyl acetate extract were separated by extraction with 3 volumes of 5 per cent. sodium bicarbonate from which the acidic substances were recovered by extraction with 3 volumes of ethyl acetate after acidifying to pH 3·0 with phosphoric acid.

Basic substances were extracted from similar plant tissues by macerating with ethyl acetate at pH 7·0 and separating as described. The basic substances in the extract were purified by partitioning with 0·01 N. hydrochloric acid, from which they were recovered with ethyl acetate after neutralizing.

Separation of extracts by paper chromatography. The ethyl acetate extracts were concentrated under reduced pressure and separated on paper chromatograms, ascending method, with isopropanol : ammonia solution, S.G. 0·88 : water (9 : 1 : 1). Chromatograms were air-dried and marker spots of GA were located under U.V. radiation after soaking that portion of the paper in 70 per cent. sulphuric acid (Phinney *et al.*, 1957). IAA was located by spraying with ferric chloride/perchloric acid reagent, and Kn by spraying with cysteine/sulphuric acid reagent and heating to 85° C. (Buchanan, 1951). The strips of paper containing the chromatograms of the extract were each cut horizontally into 10 segments, corresponding to intervals of 0·1 Rf value. Each segment was placed in a beaker with 10 ml. of 1 per cent. sucrose and 10 etiolated dwarf bean leaf disks or 10 ml. of water and 10 pea epicotyl or wheat coleoptile sections and kept in darkness at 25° C. for 24 hours.

To test these extraction procedures, 50μg. of GA was partitioned between ethyl acetate and 5 per cent. sodium bicarbonate, and 50μg. Kn between ethyl acetate and 0·01 N. hydrochloric acid. When the concentrated ethyl acetate fractions were separated on a chromatogram, GA was absent, showing that extraction was complete; but about 10μg. of Kn was located on the chromatogram of the ester fractions.

RESULTS

Growth substances found on chromatograms of extracts of cotyledons and primary leaves of dwarf bean plants. When assayed with etiolated dwarf bean leaf disks, chromatograms of extracts of the acidic substances from cotyledons and primary leaves of plants grown in controlled environment always contained a growth-promoting substance, with an Rf value similar to that of the GA marker spot. In Fig. 2 the mean diameter of the disks, measured perpendicular to the vein, which normally crossed each disk, is plotted as a histogram against the Rf value of the segment of the chromatogram. Horizontal dotted lines are drawn on both sides of the control lines at a distance equal to three times the standard error of the mean. Also, Fig 2 shows equal portions of the same primary leaf extract, separated on the same paper and assayed by wheat coleoptile and pea epicotyl sections. Growth promotion in the region corresponding to the IAA marker spot occurred on both these chromatograms. On the chromatogram assayed with coleoptile sections growth promotion also occurred close to the starting line and solvent front and a zone of growth inhibition was present between Rf values 0·7 and 0·8.

The chromatograms of the non-acidic fraction, which were left after removal of acidic substances by sodium bicarbonate and could be expected to contain neutral substances and possibly some basic ones, did not promote growth when assayed with leaf disks. There was a zone of growth inhibition

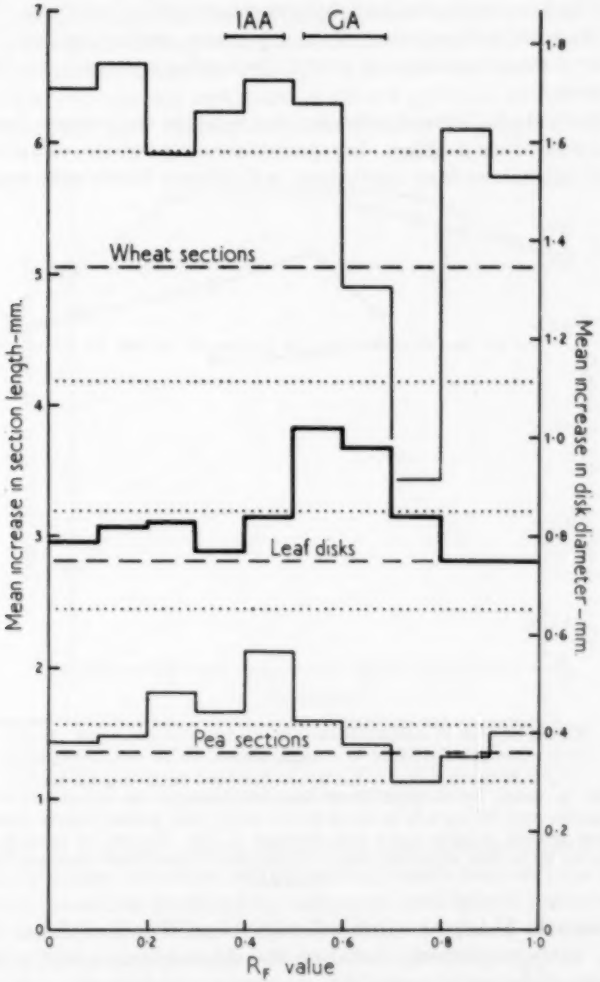

FIG. 2. Assay of chromatograms of the fraction of dwarf bean primary-leaf extract, containing acidic substances, by 6 mm. diameter etiolated dwarf bean leaf disks, 10 mm. pea epicotyl sections, and 10 mm. wheat coleoptile sections. Growth of the controls in each test is indicated by a broken line, on either side of which 3 times the standard error of the mean is indicated by dotted lines. Horizontal lines at the top of the figure indicate positions of the marker spots.

close to the starting line. Chromatograms of extracts containing basic substances only promoted growth slightly when assayed by leaf disks. This effect was not always significant, even at the 5 per cent. probability level, and there was never a clear indication of growth promotion corresponding to the Kn marker spot.

Changes in the leaf-growth substances in cotyledons and primary leaves during growth of dwarf bean seedlings. In a preliminary experiment, extracts containing acidic substances from cotyledons and primary leaves were separated on

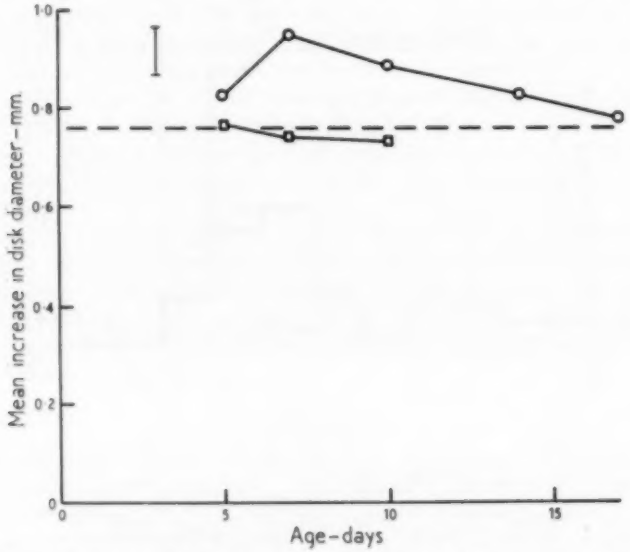

FIG. 3. Assay, by etiolated dwarf bean leaf disks, of the leaf-growth substances, with Rf 0·5–0·8, in extracts of 5 dwarf bean primary leaves during their growth in light (–o–) and darkness (–□–). Growth of controls is shown by broken horizontal line. Vertical line is three times standard error of mean.

chromatograms which were assayed with dwarf bean leaf disks. The only growth activity consistently found on the chromatograms had a similar Rf value to the GA marker spot. The chromatograms were run and assayed in pairs, so all the results could not be statistically analysed together. In a second experiment all the leaf extracts were run on one paper and only the zones of the chromatograms, Rf 0·5–0·8, corresponding to the GA marker spot, were assayed. Each of these zones was halved vertically for duplication of assay. The entire assay was completed at one time and the results were analysed together (Fig. 3). Extracts of the primary leaves were started at 5 days, when the folded leaves were removed from between the cotyledons, and continued to the 17th day, when the primary leaves had reached maximum size. Ten

leaves were extracted on each occasion as it was considered more important to obtain information about changes of growth substance per leaf than per unit weight of tissue. The growth promotion on the zone assayed increased to a maximum at the 7th day and fell close to that of the controls by the 17th. This variation with age was significant at the 0·1 per cent. probability level. Similar extractions were made from leaves produced by the same grade of

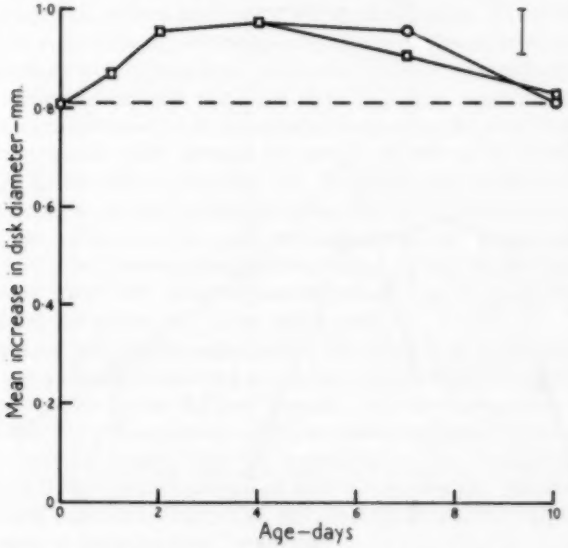

FIG. 4. Assay, by etiolated dwarf bean leaf disks, of the leaf-growth substances, with Rf 0·5–0·8, in extracts of 10 dwarf bean cotyledons during the growth of seedlings in light (–○–) and darkness (–□–). Other conventions as in Fig. 3.

seeds grown in darkness. These extractions were continued until the 10th day, when the plants were slimy and the primary leaves were still unexpanded. The growth promotion produced by extracts of dark-grown leaves was close to that of the controls. When analysed with the results of the assay of the light-grown leaves, the light effect was significant at the 0·1 per cent. probability level and the interaction between the light and age effects was significant at the 5 per cent. level.

Changes in the leaf-growth substances extracted from the cotyledons were similarly studied in a separate experiment (Fig. 4). Twenty cotyledons were extracted on each occasion. Extracts at first were made only from dark-grown cotyledons, as cotyledons did not emerge above ground until the 5th day. By the 10th day the cotyledons had withered and started to fall off and on the 14th day only 3 out of a possible 24 remained on the plants in the light cabinet. The extract of 10 entire, ungerminated seeds showed no growth-

promoting effect in the zone assayed. The growth promotion by both light- and dark-grown cotyledons reached a maximum between the 2nd and 7th days and then fell. The change with time effect was significant at the 0·1 per cent. probability level but there was no significant difference between the growth promotion by extracts from light- and dark-grown cotyledons.

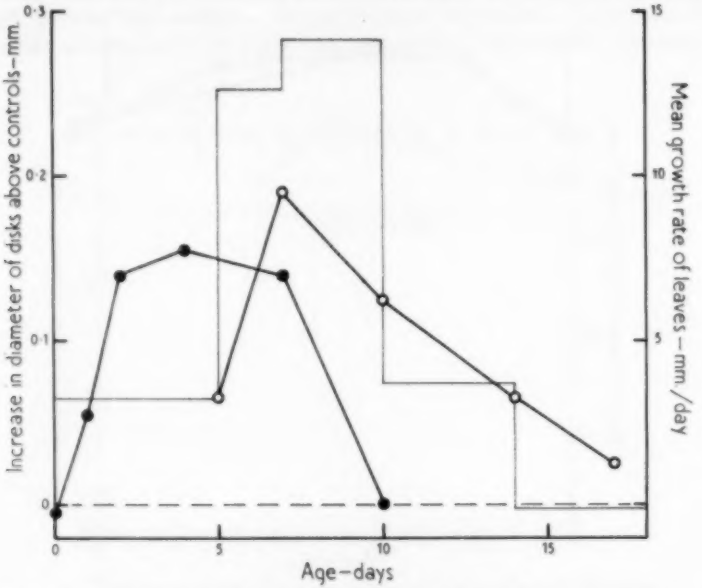

FIG. 5. Changes with age in content of leaf-growth substances in cotyledons (–●–) and primary leaves (–○–) of light-grown dwarf bean plants and in the rate of growth (histogram) of their primary leaves. Size of primary leaves was measured across breadth of leaf.

DISCUSSION

Plant extracts suspected to contain substances with a gibberellin-like growth activity are normally tested for their ability to promote internode extension in the main axis of genetically dwarf plants of peas, beans, or maize, but for the assay of leaf extracts containing unknown growth substances methods based on the growth of leaf material seem more suitable. The test with disks of etiolated dwarf bean leaves proved a suitable assay method for GA and Kn and to be unaffected by any IAA that may be in the extract.

The substance promoting growth of the dwarf bean leaf disks in cotyledon and young primary leaf extracts had on chromatograms a similar Rf value to the GA marker spot. The presence of this substance in the aqueous sodium bicarbonate fraction of the extract in which organic acids would be soluble as the sodium salt indicated that this substance was an organic acid. The con-

siderable activity shown in the dwarf bean leaf disk test, compared with the slight activity in the wheat coleoptile and pea epicotyl section tests, suggested gibberellin-like rather than auxin-like growth activity.

The maximum content in the 1·8 g. sample of leaves at the 7th day was equivalent to 0·1μg. GA (50μg. GA/kg.), derived from calibration of the dwarf bean leaf disk test with GA, and in the 5·4 g. sample of cotyledons at the 4th day was equivalent to 0·1μg. GA (20μg. GA/kg.). Radley (1958) reported 0·4μg. GA in an extract from 250 g. of tall peas. As the bean plants from which the samples of cotyledons and primary leaves were extracted in these experiments were grown from seeds within the same weight range under controlled environment with the same light intensity, quality and duration and the same temperature, it is permissible to present the data from the two experiments, together with changes in rate of growth of the primary leaves measured across breadth of leaf (Fig. 5). The maximum content of the leaf-growth substance in the cotyledons preceded that of the primary leaves which was closely associated with the maximum growth-rate of the primary leaves. The close correlation between the maximum content of leaf-growth substance of the primary leaves and their maximum growth-rate suggests that the extracted leaf-growth substance causes leaf growth.

The amount of leaf-growth substance in the cotyledons was independent of light. Growth substance occurred in primary leaves kept in light but not in darkness, where the leaves did not expand. All the leaf-growth substance that accumulated in primary leaves could not have originated in the cotyledons because the leaves had more than the cotyledons, a fact emphasized because 10 leaves were extracted and compared with 20 cotyledons. Moreover, at the peak of growth-substance content in the primary leaves the cotyledons still contained most of their activity.

These experiments provided no evidence for the occurrence of more than one leaf-growth substance. However, the Rf values of different gibberellins are similar (Bird and Pugh, 1958), although the gibberellins vary in biological activity (Brian, 1959), so what has been attributed to changes in concentration of one substance may be partly or entirely attributable to chemical changes in the growth substances.

ACKNOWLEDGEMENTS

I wish to thank Dr. D. J. Watson and Dr. E. C. Humphries for helpful discussions during this work.

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Some Responses of *Avena* Coleoptiles to Ethylene

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SUMMARY

Ethylene pretreatment of intact *Avena* seedlings or of excised coleoptile sections results in an increased response of the cells to auxin. It is suggested that ethylene brings about the acceleration of hydrolytic reactions controlling the physical properties of cell walls and hence increases their capacity for growth. Coleoptile elongation of intact seedlings is inhibited by ethylene; this inhibition is concurrent with a lateral expansion of the entire coleoptile. It is suggested that under a given set of conditions coleoptile cells are capable of attaining a finite volume and that the preferential lateral expansion induced by ethylene is accomplished at the expense of longitudinal extension. Experiments with intact and deseeded plants indicate that lateral expansion depends on the supply of some factor from the endosperm.

INTRODUCTION

EVER since Neljubow (1911) described the 'triple response' of pea epicotyls to ethylene a variety of responses induced by this unsaturated hydrocarbon have been reported but only a few mechanisms have been suggested, mainly based on some effect on the auxin metabolism of the plant, to explain its morphological effects (Van der Laan, 1934; Michener, 1938; Borgstrom, 1939). However, it is possible to explain these effects through some influence on metabolic mechanisms other than those directly involved in auxin synthesis and destruction; for example, the reactions that control the physical properties of cell walls. The work described in this paper brings some indirect evidence to support this idea. The original observations by Michener (1938) on the increased response of cells to auxin following ethylene pretreatment of the coleoptile have been extended and some new data are presented on the inhibition of elongation and the concurrent lateral expansion of intact coleoptiles during ethylene treatment.

METHODS

Avena sativa (var. Victory) seeds were selected for uniformity in size, dehusked, soaked in distilled water for 2 hours and then mounted on glass plates lined with filter-paper. These plates were supported at a 60° angle in glass dishes and water added to a level 5 mm. below the tip of the embryonic root. The seedlings were grown in a darkroom at 24° C. and exposed to red light for the first 24 hours of growth. All subsequent operations were carried out in red light.

The section test was performed by cutting a single 3 mm. section 3–4 mm. below the tip of each coleoptile at the age specified in each experiment. These sections were randomized, floated in petri-dishes containing 15 ml. of the test solution and grown in darkness. The sections were measured with a binocular microscope equipped with an eye-piece micrometer scale.

Ethylene treatments were generally carried out in 17-litre glass jars; however, for the determination of the time-course of elongation in the presence of ethylene the sections were placed in petri-dish chambers in which it was possible to measure the sections under red light without removal from the ethylene atmosphere. The chamber consisted of the lower half of a 10-cm. petri-dish covered with a glass plate sealed on with a vacuum grease. The plate was fitted with a rubber stopper through which passed two glass tubes; the outer ends of these tubes were provided with rubber tubing and glass rod plugs. The desired ethylene concentration in the chamber was achieved by connecting one of the tubes to a 500 c.c. flask filled with a known mixture of air and ethylene and flushing the entire volume of the gas through the chamber by displacement with water. In all experiments the concentration of ethylene was 0·1 per cent. Within the chamber the coleoptile sections were floated between parallel glass rods or threaded on thin glass needles.

The volumes of coleoptile sections were obtained after removal of the primary leaves, blotting, and withdrawal of water from inside the cylinders by means of a fine glass capillary connected to a suction pump. The sections were then introduced into a vertically mounted glass tube of uniform bore containing water with a trace of detergent which ensured the entry of water into the cylinder. The displacement of the water was measured by means of a cathetometer equipped with a millimetre scale and vernier. Calibration of the tube indicated that changes in volume less than 1 per cent. of the original value of an individual section could be determined.

Indole acetic acid solutions (IAA) were unbuffered; they were stored in a refrigerator and renewed at least once a fortnight.

RESULTS

Michener (1938) reported that ethylene pretreatment of intact *Avena* seedlings increased the subsequent response of the coleoptiles to auxin in both the curvature and the section tests. It is quite possible that the increased response in the section test may be partly due to the greater number of cells per unit length of coleoptile (Table I) that results during the ethylene-induced inhibition of elongation (Michener, 1938; and Table II) without individual cells being necessarily stimulated to greater growth.

The possibility that ethylene may actually increase the growth capacity of individual cells was explored by using sections with the same number of cells. This was ensured in two ways: (*a*) Sections were obtained from seedlings that had been placed in ethylene when they were older than 96 hours, since after that age the number of cells per section from both control and treated coleoptiles must be the same (Tables I and II); and (*b*) by applying

the ethylene pretreatment to excised sections from air-grown coleoptiles when, again, all sections can be assumed to contain the same number of cells.

TABLE I

Cell number per unit length of the inner epidermis of coleoptiles from intact seedlings placed in ethylene at various ages and measured when 120 hours old

Age (hrs.) at beginning of treatment	Cell number
24	360
48	380
72	325
96	230
120 (control)	220

TABLE II

Growth inhibition of 120-hour-old Avena *seedling coleoptiles by exposure to ethylene starting at different ages after beginning of soaking*

Age (hrs.) at beginning of treatment	Coleoptile length in millimetres measured at (hrs.)			
	48	72	96	120
48	4	10	18	20
72	—	15	23	24
96	—	—	34	36
Control	—	—	—	37

TABLE III

The effect of a 24-hour ethylene pretreatment of intact Avena *seedlings on the subsequent elongation of coleoptile sections in water and auxin. Each value represents the mean of 20 sections after 24 hours of growth in the solutions*

Age (hrs.) at beginning of treatment	24 hrs. pretreatment in	Age (hrs.) at cutting of sections	Per cent. Elongation (24 hrs.)	
			Water	1×10^{-5} M. IAA
96	Air	120	7	32
	Ethylene		14	55
120	Air	144	1	7
	Ethylene		2	14

The first approach is illustrated by results in Table III. In this experiment intact seedlings were treated with ethylene for 24 hours starting at 96 and 120 hours, then sections were cut and floated on water or 1×10^{-5} M. IAA and measured 24 hours later. It is seen that even in the case of older plants ethylene pretreatment roughly doubled the subsequent elongation of sections as compared with sections from non-treated coleoptiles.

Typical results of the second type of experiment are presented in Table IV. Sections were cut from plants 75 hours old and equal numbers placed in two dishes with water; 25 sections from each dish were measured and then one dish was placed in ethylene while the other was left in air. Twenty-four hours later the sections from each pretreatment were randomized into two lots of 25 sections each, measured to obtain the extent of elongation during pretreatment, and then from each pretreatment one lot was placed in water and the other in 1×10^{-5} M. IAA. The lengths were measured again 24 hours later. This experiment showed that ethylene-pretreated sections transferred to auxin elongated more than air-pretreated sections, while there was no response in water. This increased response to auxin caused by ethylene pretreatment of sections was never large (growth was usually between 10 and 15 per cent. greater than in controls) but it was found consistently.

TABLE IV

Elongation of coleoptile sections in the presence or absence of ethylene and effect of this pretreatment on the subsequent elongation of these sections

		Length (micrometer units)			Elongation following pretreatment	
Pre-treatment	Lot	Initial (75 hrs.)	End of pretreatment (99 hrs.)	Conditions following pretreatment	Length in micrometer units (149 hrs.)	Per cent. elongation following pretreatment
Air	1	52·5	75·5	Air—Water	87·1	15·3
	2		71·9	Air—Auxin	103·8	44·3
Ethylene	3	52·4	70·1	Air—Water	79·0	12·6
	4		69·4	Air—Auxin	111·5	60·6

Since in both types of experiment the sections presumably contained the same number of cells at the end of the pretreatment it follows that the growth capacity of individual cells was increased by the ethylene pretreatment, and that this increase was independent of other ethylene-induced reactions in the rest of the seedling; this is borne out by the results after pretreatment of excised sections.

A single measurement after a prolonged period of growth does not always adequately describe the behaviour of a tissue (Audus, 1952; Marinos, 1957); for example, the increased response following ethylene pretreatment may be the result of a greater initial rate of elongation and/or extension of the growth period. Therefore, the time course of elongation in water or auxin following ethylene pretreatment of intact seedlings, of excised sections, and of sections elongating in the presence of ethylene, was determined. Details and results of representative experiments are shown in Fig. 1.

From Graphs A and B it can be concluded that the greater ultimate length of ethylene pretreated tissues is primarily the result of an increased initial elongation rate; however, there is also some indication, particularly in Graph A, that the period of growth is extended following ethylene pretreatment.

Graph C shows that the time-course of elongation for sections growing in air or in the presence of ethylene is identical, at least for the first 24 hours of growth, in both water and auxin.

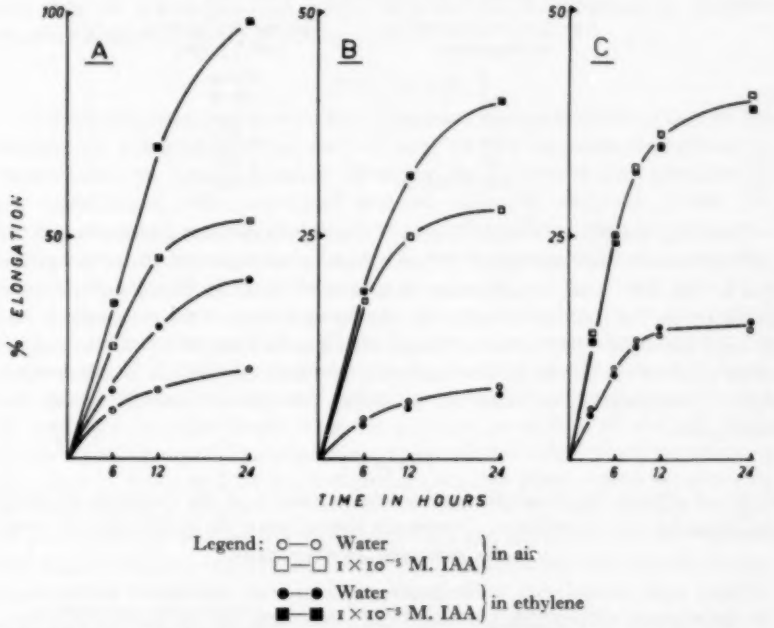

FIG. 1. Time-course of coleoptile section elongation under different conditions of ethylene treatment.

Graph A. *Pretreatment of intact* Avena *coleoptiles*
Seedlings left in air or placed in ethylene when 72 hours old.
Treatment terminated 24 hours later when sections were cut and floated in water or auxin.

Graph B. *Pretreatment of* Avena *coleoptile sections*
Sections obtained from 72-hour-old seedlings floated in water and treated with air or ethylene for 24 hours, and then transferred to water or auxin. Elongation is expressed as per cent. increase over the value at the end of pretreatment.

Graph C. Avena *coleoptile sections growing in the presence of ethylene*
Sections obtained from 88-hour-old seedlings and their elongation followed in petri-dish chambers.

Michener (1938) also reported that oat coleoptiles failed to develop swellings which characterize the response of other species to ethylene. In the course of this work it was found that this is true in the sense that distinct localized swellings do not occur in *Avena* coleoptiles but it overlooks the fact that during growth in ethylene there is a considerable increase in the diameter of the entire coleoptile. This is shown by the results in Table V, where increases in girth have been measured in terms of the volume of tissue per unit length.

TABLE V

Changes in volume/unit length in the coleoptile of intact Avena *seedlings placed in ethylene at various ages and measured when 120 hours old. The values represent the mean of 20 sections per treatment*

Age (hrs.) at beginning of treatment	Volume per section mm.^{3}/3 mm.
3	6·000
24	5·816
48	5·557
72	4·853
96	4·205
120 (control)	3·890

From the results in Tables II and V it is apparent that ethylene modifies cell expansion in the coleoptile of intact *Avena* seedlings: it inhibits elongation and at the same time it stimulates transverse expansion. These experiments, however, do not indicate whether the lateral expansion of the coleoptile is due to some localized effect on the coleoptile itself or to some effect of ethylene on other parts of the seedling. For example, ethylene may induce the formation and/or translocation of materials necessary for lateral expansion from the endosperm.

TABLE VI

Effect of ethylene on elongation and lateral expansion of the coleoptile of intact and deseeded Avena *seedlings. Treatments started when the plants were 72 hours old and measured 48 hours later*

		Elongation		Lateral expansion	
Treatment		Coleoptile length (mm.)	% inhibition due to ethylene	Volume/3 mm. section (mm.^{3})	% increase due to ethylene
Air	Intact	39·3	—	3·434	—
	Deseeded	27·6	—	2·923	—
Ethylene	Intact	30·7	21·9	4·144	+20·5
	Deseeded	24·1	12·7	2·997	+2·5

This possibility was explored by determining the effect of ethylene on elongation and lateral expansion of the coleoptiles of intact and deseeded seedlings. Seventy-two-hour old seedlings were selected for uniformity of coleoptile growth and divided into lots of 20 seedlings each. Two lots were left intact while another two were deseeded. The coleoptiles of each lot were measured and then one lot of intact and one of deseeded seedlings were left in air while another identical set was placed in ethylene. When the seedlings were 120 hours old the coleoptiles were measured again and a section was obtained from the middle of each coleoptile for the determination of the volume per unit length (preliminary estimations indicated that a 3 mm. section obtained from the middle of a coleoptile gave a representative estimate of the volume per unit length of the entire coleoptile). The results are shown

in Table VI. Coleoptile elongation was inhibited by ethylene in both intact and deseeded plants but the effect was greater in the case of intact seedlings; also, lateral expansion was induced in the coleoptiles of intact seedlings but not in deseeded coleoptiles. Other experiments with isolated sections showed that they do not respond by lateral expansion in the presence of ethylene whether floated in water or auxin.

DISCUSSION

The results presented here confirm Michener's original observations on the stimulatory effect of ethylene pretreatment on the subsequent response of *Avena* coleoptile tissues to auxin. However, the additional data provided by the experiments with pretreated isolated coleoptile sections (Table IV, Fig. 1B) warrant a reconsideration of his hypothesis that ethylene must cause 'the activation or accumulation of a food factor or some other factor promoting growth'. In the case of excised sections all possible sources of the postulated food factor have been removed and any effects of ethylene must be exerted on the materials present in the cells at the time of excision. Consequently the mechanism of increased response must be localized in the coleoptile itself. One possibility is that increased growth may result through the acceleration of enzymatic reactions controlling the physical properties of the cell walls (Frey-Wyssling, 1950). Investigations on the effect of ethylene on the ripening processes in fruits and on the metabolism of other plant organs support this proposal (*vide*, e.g., Harvey, E. M., 1915; Harvey, R. B., 1938; Hansen, 1939). Of particular relevance are the findings of Hall and Lane (1952), who reported that during ethylene-induced abscission in cotton the structural carbohydrates, proteins, and the insoluble nitrogen fraction in the leaves were rapidly hydrolysed while at the same time there was a considerable stimulation of respiratory enzymes. A similar stimulation of hydrolytic enzymes in coleoptiles exposed to ethylene could result in the 'softening' of the cellulosic and pectinaceous constituents of the cell walls and so increase the growth capacity of the cells.

This explanation, however, is faced with one difficulty. From the results (Table IV and Fig. 1C) it is seen that excised sections grow identically for the first 24 hours whether grown in air or in the presence of ethylene although those from ethylene may grow more later (Table IV). If ethylene affected cells in the proposed manner then why is it that sections do not grow more in its presence than they do in air? One suggestion is that changes in cell wall properties are not instantaneous upon exposure to ethylene but reach a critical level sometime after the first 24 hours. This proposal needs further testing by following the growth of sections in the presence of ethylene over longer periods of time. It is interesting however, that Hall and Lane (1952) have observed a delayed effect of defoliants, including ethylene, on the acceleration of hydrolytic reactions in the cotton leaf.

One of the more prominent effects of ethylene on plants is the induction of lateral expansion. Van der Laan (1934) postulated that ethylene-induced

swellings in pea epicotyls are due to some condition limiting their longitudinal extension. This suggestion is not supported by the present results (Table VI), where it is shown that in deseeded plants inhibition of elongation is not accompanied by lateral expansion, nor is it supported by many other cases where elongation can be inhibited without a corresponding lateral expansion. This does not preclude the possibility of a causal relationship between elongation and lateral expansion, but it is probably the reverse from the one suggested by Van der Laan. It is a reasonable assumption that a cell is capable of a finite increase in *volume* under a given set of conditions; therefore, if lateral expansion is preferentially stimulated the cells will grow in diameter at the expense of length. The fact that lateral expansion can take place only in the presence of the endosperm suggests that ethylene must cause the formation and/or translocation of materials originating in the endosperm and necessary for lateral expansion. On this basis the absence of a prominent growth inhibition in isolated sections could be attributed to the lack of factors necessary for lateral expansion and the freedom of the cells to expand longitudinally.

It may be surmized from the foregoing that the inhibition of elongation induced by ethylene is qualitatively different from the inhibitions induced by metabolic inhibitors or antiauxins. In the latter cases elongation ceases because there is a block in metabolic reactions, while in the case of ethylene it is, at least partly, a secondary effect resulting from the concurrent expansion in the lateral direction. In the literature, expansion in the longitudinal axis has become synonymous with the total phenomenon of cell expansion which involves growth in both the longitudinal and transverse directions. The work presented here suggests that the direction of expansion may be controlled by specific factors; what these factors are is unknown at present. This idea gains support from the work of Galston and co-workers (1953) who found that at certain concentrations benzimidazole inhibits the elongation of pea epicotyl sections while it stimulates their lateral expansion; apparently, in this case also, preferential expansion in the transverse direction is accomplished at the expense of elongation.

The author wishes to express his thanks to Dr. L. H. May for his helpful criticism during the preparation of this paper.

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The Production of the *Striga* and *Orobanche* Germination Stimulants by Maize Roots

I. THE NUMBER AND VARIETY OF STIMULANTS[1]

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SUMMARY

By means of paper partition chromatography and a suitable biological test, the number and variety of the *Orobanche* and *Striga* germination stimulants produced by maize roots have been assessed. It is shown that extracts of maize roots and aqueous solutions in which roots have been suspended (diffusate solutions) contain a complex of stimulating substances consisting of, at least, one water-soluble and a number of ether-soluble stimulants. Germination of *O. minor* is promoted by the water-soluble stimulant and one of the ether-soluble stimulants (M_o). Germination of *S. hermonthica* is promoted by another ether-soluble stimulant (M_s). Aqueous extracts are rich in M_s and the water-soluble material, whereas the diffusate contains a higher proportion of M_o and only a trace of the water-soluble stimulant.

The complex of ether-soluble stimulants, but not the water-soluble stimulant, accumulates in a solution of glucose when it is exposed to fragments of maize roots. These solutions contain a high proportion of M_o which is synthesized by the fragments and passes out into the sugar solution. It is suggested that the water-soluble stimulant is a precursor of M_o.

The effects of the water-soluble stimulant and of M_s and M_o are greatly enhanced if a trace of the diffusate solution from linseed roots is mixed with them. The significance of the enhancement is discussed.

INTRODUCTION

THE seeds of angiospermous root parasites usually germinate only after exposure to stimulating substances. For many species such as *Orobanche minor* and *Striga hermonthica* the substances escape not only from the growing roots of the few hosts which they parasitize, but also from those of many other plants. Root secretions of *Linum usitatissimum* and *Sorghum vulgare*, for example, stimulate both species and it has been shown by Brown and his colleagues that the stimulating substances from these plants have many similar properties. They are both lipophilic materials, readily soluble in organic solvents, and they travel on a paper chromatogram developed in a butanol:acetic acid system to about Rf 0·9 (Brown, Greenwood, Johnson, Long, and Tyler, 1951; Brown, Johnson, Robinson, and Tyler, 1952).

[1] The material of this paper and the next form part of a Thesis presented to the University of Leeds for the degree of Ph.D.

In the investigation described here the stimulating substances for *O. minor* and *S. hermonthica* produced by *Zea mays* have been examined chromatographically using three different preparations:

1. aqueous and ether extracts of seedling roots,
2. an aqueous solution in which seedling roots have been suspended (diffusate solution), and
3. a solution of glucose in which fragments of seedling roots have been suspended and shaken for various times (segment fluids).

MATERIALS AND METHODS

Preparation of the stimulating solutions. Extracts and segment fluids were prepared from primary roots of *Zea mays* (var. Giant white horse tooth). Seeds were germinated on filter-paper at 25° C. in the dark. After 48 hours roots that were between 2 and 3 cm. in length were selected and, from these, fragments 3 mm. in length were excised using the apparatus described by Brown and Broadbent (1951).

Aqueous extracts of the fragments were made by freezing groups of them in water at −10° C. for 1 hour. After thawing, the water was left in contact with the tissue for a further hour, when the fluid was removed and applied immediately to the chromatogram. Ether extracts were made by immersing the fragments in ether for 1 hour, after which the ethereal solution was removed, evaporated *in vacuo* and the residue dissolved in water.

Segment fluids were prepared by suspending 20 fragments in 2 ml. of the sugar solution and shaking at a constant speed for periods up to 24 hours at 25° C. in the dark. The fluids were then separated from the tissue and applied immediately to the chromatograms.

The diffusate solution was prepared by suspending maize seedlings over distilled water. A circular cylinder of stainless steel with muslin stretched over one end was placed in a jar containing 50 ml. distilled water. The cylinder was of such a height that the level of the water just reached the muslin. Five maize seedlings, germinated as described above, were placed on the muslin with their roots projecting through into the water. The jar was then closed and allowed to stand in the dark for 4 days at 25° C. After removal of the seedlings the liquid was stored at pH 4 in a refrigerator. Under these conditions it retained its stimulating effect on the seeds for several months.

Chromatographic techniques. Chromatograms were prepared on Whatman No. 1 filter-paper and developed in a descending flow of solvent for 15 hours. The stimulating solutions were applied along the starting line as a band 1 cm. wide and dried. The choice of solvents was somewhat limited. Alkaline solvents were avoided because of the instability of the stimulants in alkaline solution (Brown *et al.* 1951, 1952) as were solvents likely to interfere in the subsequent biological test. A mixture of tertiary butanol 4:ethanol 4:water 2 was found to be a satisfactory system.

Side strips of the developed chromatograms were removed and examined

both by ultraviolet light and by suitable spray reagents. Sugars were detected either by aniline hydrogen phthalate (Partridge, 1949) or by p-anisidine phosphate (Mukherjee and Srivastava, 1952); amino-acids by ninhydrin; and phenols by bis-diazotized benzidine (Linstedt, 1950).

In the absence of a suitable chemical or physical test the presence of stimulating substances on those portions of the chromatograms that were of interest was detected by means of the parasite seeds. The papers were cut transversely into strips each 1 cm. wide. These were then placed individually in small tubes containing the eluting fluid and the tubes were left at 4° C. for 4 hours. Percentage germination of the parasite seeds in each eluate was then assessed by the method described below. In most cases the strips were cut in half longitudinally; one series was eluted with water and the other with a stimulating solution of standard activity. As a standard, the diffusate solution from roots of linseed was used diluted a hundred times (Brown, Greenwood, Johnson, and Long, 1951).

The biological test. Parasite seeds were used only after storage in favourable conditions of moisture and temperature. The seeds were scattered thinly on moistened sintered-glass disks contained in petri-dishes, and incubated at 25° C. After about 21 days the seeds of *O. minor* were washed from the disks in distilled water at 25° C. and transferred to clean disks, and this was repeated at regular intervals in order to reduce the growth of fungi and other micro-organisms. As seeds of *O. minor* moisture-stored at 25° C. for 21 days retain their maximal response for a long period it was possible to use seeds of the same sample for a number of experiments (Brown *et al.*, 1951). On the other hand, seeds of *S. hermonthica* do not retain their maximal response with prolonged storage. The peak response of the seeds used here was reached after about 12 days at 25° C. Thus a fresh sample of seeds kept moist for 12 days was used for each experiment.

Percentage germination was determined in multiple hanging-drop cultures. The method was adapted from that described by Brown *et al.* (1951). In the earlier method drops of different solutions were all sealed in a common chamber in which coalescence of drops could occur. This difficulty was eliminated by sealing the replicate drops of each solution in separate chambers. The basal glass plate used by Brown *et al.* was replaced by an aluminium plate about 6×3·5 in., having 5 depressions 2·8×0·8×0·125 in. deep each slightly smaller than a microscope slide. The plates were made from sheets of pure aluminium (soft metal, 24 gauge) and were pressed in a steel die which had been designed specifically for the purpose.

Six drops (0·01 ml.) of each solution to be tested were placed on a clean microscope slide; 25 to 50 seeds were introduced into each drop by means of a platinum loop, and the slide was then inverted over one of the depressions in the basal plate. Five solutions were treated in this manner and when all the slides were in position, they were sealed to the plate with a molten mixture of equal parts of vaseline and embedding wax. In order to obtain uniform drops and to avoid coalescence, each slide was polished before use

with a cotton pad bearing a trace of DC 200 silicone and heated on a hot plate for several minutes. Plates bearing seeds of *S. hermonthica* were incubated at 35° C. for 2 days and those bearing seeds of *O. minor* for 5 days at 25° C. Each value recorded below is the mean of the six replicate determinations.

EXPERIMENTAL RESULTS

In the description of the results only those zones of stimulation which have been observed consistently after a number of trials are considered. Small sporadic germinations are ignored as are minor fluctuations above and below the control value of the standard eluting fluid where these have shown no consistent pattern.

Extracts. The responses of the parasite seeds towards the chromatograms of the ether and aqueous extracts are shown in Figs. 1 and 2 respectively. It is evident from Fig. 1 that the stimulating substances in the ether extract travel in a zone with Rf limits of about 0·75 to 0·95. Not all the eluates from this zone, however, germinate both species. This only occurs in a narrow zone from Rf 0·85 to 0·95. The eluates at Rf 0·75 to 0·85 germinate the seeds of *Striga* only. The peak response of *Orobanche* is situated at about Rf 0·92 while the peak for *Striga* is slightly in advance of this at about Rf 0·87.

The data of Fig. 2 also show that the pattern of germination of the two species is different. With respect to *Orobanche* two major zones of stimulation are now evident, one near to the solvent front with the peak response at Rf 0·90 and a second, more prominent zone, with the peak at Rf 0·60. The former is probably due to the same material that is present in the ether extract but the latter is confined to the aqueous extract.

With *Striga* seeds, the bulk of the stimulation does not occur in the regions of *Orobanche* activity. Instead, there is one widespread zone, the most significant part of which stretches from Rf 0·50 to 0·85 with the peak response at Rf 0·70. Much of this stimulation is probably due to the same material that is present in the ether extract. In this case, however, the presence of numerous cell constituents in the extract has probably interfered with the development of the chromatogram causing the stimulating material to form a longer and more diffuse spot. Except in one instance, the stimulants occupy regions devoid of known substances, the exception being a phenolic compound which appears as a pink spot at Rf 0·80 in the zone where *Striga* stimulation is very marked. It is unlikely that the phenol is the actual stimulant because the area to which the seeds respond is much greater than that occupied by the phenol.

A further point emerges from the data of Fig. 2. It will be observed that the responses of the seeds in the stimulating zones are considerably enhanced when the standard eluting fluid is used instead of water. At Rf 0·7, for example, the response of *Striga* seeds in the eluates prepared with the standard fluid is about 30 per cent. The water eluate and the standard fluid alone, however, are each only capable of promoting a response of 1 per cent. The effect of

mixing the two is equivalent to increasing the concentration of the standard fluid a hundred times. Enhancement of the responses of *Orobanche* seeds at Rf 0·6 and 0·9 also occurs when the linseed fluid is used as eluant.

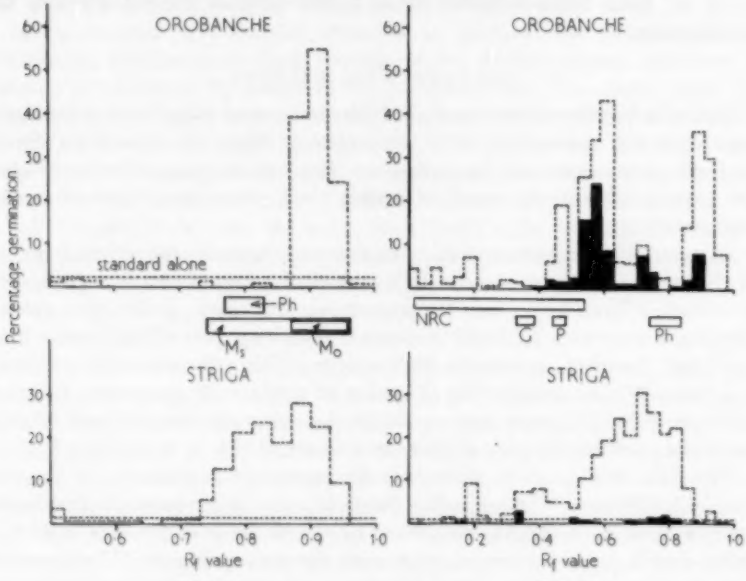

FIG. 1. FIG. 2.

FIG. 1. Histograms showing distribution of stimulating substances for *O. minor* and *S. hermonthica* on a paper chromatogram of an ether extract of maize roots after development with a solvent mixture of tertiary butanol 4 : ethanol 2 : water 2, and after elution with the standard stimulating solution. Ph-unknown phenolic compound.

FIG. 2. Histograms showing distribution of stimulating substances on a paper chromatogram of an aqueous extract of 60 fragments of maize roots excised at a distance of 3–6 mm. from the tip, after elution with water (solid) and the standard stimulating solution (dotted). G-glucose; P-pentose sugar; NRC-ninhydrin reactive compounds; Ph-unknown phenolic compound.

Diffusate solution. The results obtained with the diffusate solution are shown in Fig. 3. It is evident that the main stimulating material travels at about Rf 0·87 to 0·95. When water is used as the eluting fluid, both species respond to the same eluates. With the standard eluting fluid, however, the pattern of germination changes. With *Striga*, the control value of the standard is enhanced in a number of places along the paper, the most significant of which is at Rf 0·75 to 0·90 with a maximal response at Rf 0·88. It will also be observed in this region that a synergistic effect is promoted similar to that indicated above for the aqueous extract. Using *Orobanche* seeds, the control value of the standard is also enhanced in various regions, the most prominent of which stretches from Rf 0·55 to 0·96. Within this zone, in addition to the peak at 0·9, there is a suggestion of a second peak at about Rf 0·55.

Further data obtained with the diffusate solution are shown in Fig. 4. The

response of *Orobanche* is shown towards two chromatograms, one prepared from an ether extract of the diffusate and the other from the residue. The main stimulating substances in the diffusate which travel at Rf 0·9 are clearly

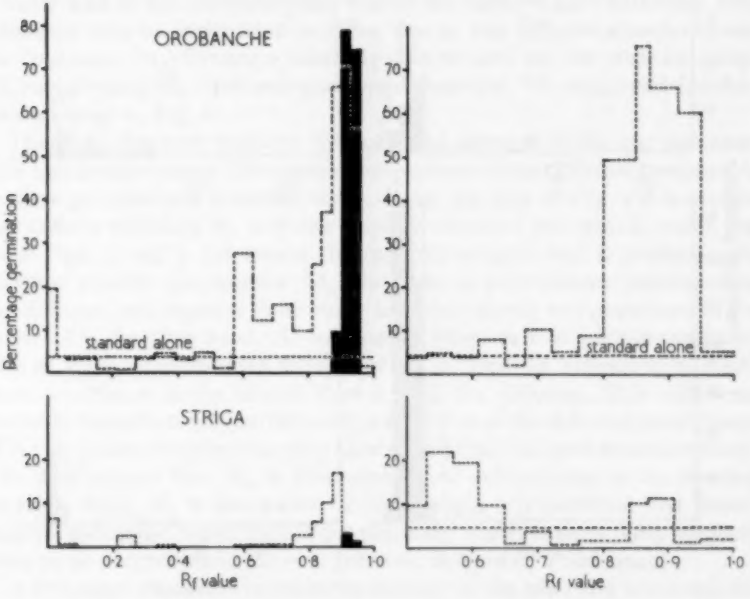

FIG. 3.

FIG. 4.

FIG. 3. Histograms showing distribution of stimulating substances on a paper chromatogram of 1 ml. of maize diffusate solution after elution with water (solid) and the standard stimulating solution (dotted).

FIG. 4. Histograms showing distribution of stimulants for *O. minor* on paper chromatograms of the ether extract of 50 ml. of maize diffusate solution (upper) and the residue (lower) after elution with the standard stimulating solution.

soluble in ether, and appear only slightly on the chromatogram of the residue. It is significant that the chromatogram of the residue bears a zone of stimulation at Rf 0·55—0·60 which does not appear on the chromatogram of the ether soluble materials. This zone is similar in position to one of the zones of stimulation in Figs. 2 and 3.

Segment fluid. Figs. 5 and 6 show results obtained with a fluid which was originally a 0·01 M. solution of glucose. It is clear that stimulation is not caused by glucose. After culture for 6 hours a stimulant for *Orobanche* appears in the sugar solution and it travels near to the solvent front giving a peak response at Rf 0·90. Its amount increases considerably during the next 6 hours. It will be observed that the control value of the standard eluting fluid is increased in this part of the chromatogram but apart from a slight effect at Rf 0·5—0·6 there is no significant enhancement elsewhere on the paper.

Stimulatory materials for *Striga* seeds also appear in the sugar solution. After 6 hours using water as eluant, there is a zone of stimulation stretching from Rf 0·55 to 0·85 with a peak response at Rf 0·85. This peak does not

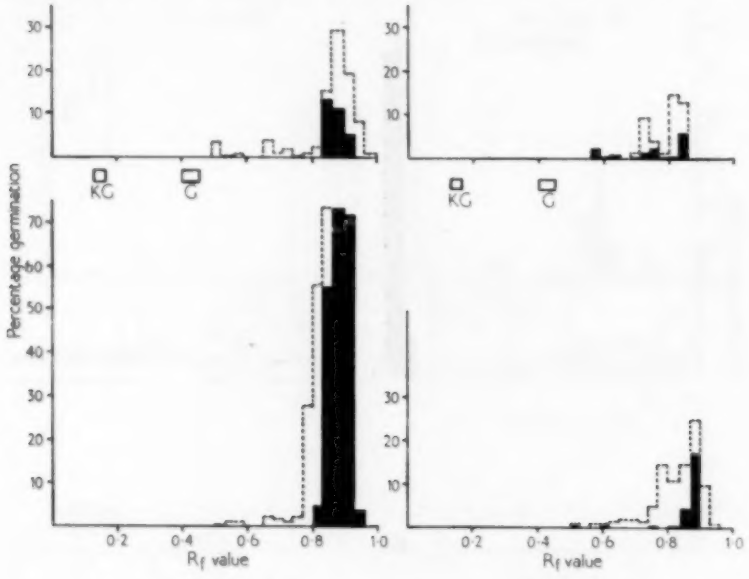

FIG. 5. FIG. 6.

FIG. 5. Histograms showing distribution of stimulating substances for *O. minor* on paper chromatograms of 1 ml. of 10^{-3} M. glucose solution in which 10 fragments of maize roots (3–6 mm.) have been shaken at 25° C. in the dark for 6 hours (upper) and 12 hours (lower) after elution with water (solid) and the standard stimulating solution (dotted). G-glucose; KG-2 ketogluconic acid.

FIG. 6. Histogram showing distribution of stimulating substances for *S. hermonthica* on the chromatograms of Fig. 5.

coincide with the peak for *Orobanche*. After elution with the standard eluant, two peaks are apparent, one at Rf 0·73 and another at Rf 0·83. With time, the quantity of the material increases.

DISCUSSION

A marked feature of the data is the similarity in the chromatographic behaviour of the stimulating materials in the three preparations and it is highly probable therefore that the same materials are involved. The data indicate that two types of stimulant are produced by maize, one which is soluble in water but not in ether, and a second type, soluble in both water and ether. The water-soluble stimulant travels with an Rf value of about 0·6 and is found in aqueous extracts of the roots and in the diffusate, but is probably absent from the segment fluid.

The ether-soluble constituents are the main stimulants produced by maize and they are present in all three preparations. The data of Figs. 1 and 2 indicate that the material towards which *Striga* seeds respond is spread over a wider area of the chromatogram than is the material for *Orobanche*. This difference may be interpreted as being due to two different stimulants, one for *Orobanche* (M_o) forming a relatively discrete spot and the other for *Striga* (M_s) overlapping M_o but forming a more diffuse spot. The suggested distribution is shown in Fig. 1.

The data obtained with the diffusate and segment fluids are consistent with this interpretation. The relative proportions of the different components in these preparations, however, varies. From the data of Fig. 2 it is evident that there is sufficient M_o to give a response of only 8 per cent. in water, but from Figs. 3 and 5 (12 hours) the quantity is such that it promotes the maximal possible germination. M_o, therefore, is a prominent constituent of the diffusate and segment fluid but it is comparatively less prominent in the extract. On the other hand, the response of *Striga* in Fig. 3 is low compared with its vigorous response in the data of Fig. 2. Thus M_s appears to be much more prominent in the extract than it is in the diffusate. This difference probably depends largely on the relative stabilities of the different constituents of the stimulant complex once they have passed from the protoplast into water. The data suggest that M_o is fairly stable and accumulates in the external medium, while M_s is less stable. In this respect it is pertinent that Brown and his colleagues found the *Striga* stimulant which escapes from sorghum roots to be very unstable (Brown, Johnson, Robinson, Todd, 1949).

A difference also exists between the balance of the ether and water-soluble constituents of the extract and diffusate. In Fig. 2 the response of *Orobanche* is greater in the zone occupied by the water-soluble stimulant than it is towards the region occupied by M_o, but in Fig. 3 the reverse is the case. Indeed in Fig. 3 the presence of the water-soluble stimulant is only revealed after eluting the chromatogram with the standard stimulating solution, indicating that only a trace of it is present. The difference in this case is probably due to the rates at which the stimulants pass out from the protoplast. It will be recalled that the permeability of the protoplast is greater towards fat-soluble than it is towards water-soluble substances. The ether-soluble stimulants are clearly highly lipophilic and will escape more readily from the roots than the water-soluble stimulant.

The presence of a number of stimulants in the maize preparations has been inferred, to a large extent, from the data obtained with the standard stimulating solution. It is evident that a considerable enhancement of seed response is obtained by mixing a trace of the linseed preparation with the maize stimulants. The enhancement is not peculiar to linseed. Enhancements of a similar magnitude to those indicated by the data of Figs. 2 and 3 are also promoted by the diffusate from sorghum roots, but they are not obtained with the diffusate from maize. It must be concluded that a constituent of the linseed and sorghum preparations interacts synergistically with the maize

stimulants. The identity of this constituent has not been fully established, but it has been found that when the ether-soluble stimulants in the linseed and sorghum preparations are destroyed either by boiling or by alkali, the ability of the solutions to enhance germination is also destroyed. This suggests that the interaction occurs between the ether-soluble stimulants in each preparation.

It might be expected therefore that the ether-soluble stimulants of linseed and sorghum differ in some way from those of maize although, with the system used here, it is difficult to distinguish them chromatographically. But if this is so, it will not account for the synergistic effect. It is considered that germination of the parasite seeds is dependent not on one discrete ether-soluble stimulant but on a number all of which may be very similar in nature. The linseed diffusate provides at least one of the required materials, and so promotes a greater effect by raising the limiting concentration of that material. This implies that linseed, sorghum and maize may all produce a similar complex of stimulants differing only in the particular balance of the constituents, and it is conceivable that M_s and M_o may consist of more than one substance.

Finally, it has been shown above that the ether-soluble stimulants appear in the external fluid when isolated fragments of maize roots are cultured in a solution of glucose. The observation that a glucose solution can be rendered stimulatory in this way was first made by Brown and his colleagues at Leeds. Robinson (1951) reported that the fragment excised at a distance of 1·5 to 3·0 mm. from the tip, when immersed in a glucose solution, produced a stimulant for *Striga* seeds and at the same time Greenwood found that these solutions germinated seeds of *O. minor*. The present series of data have been obtained with a segment of root slightly behind that originally used because the segment excised at a distance of 3·0 to 6·0 mm. from the tip gives more active preparations. The data of Fig. 2 show that this segment contains the ether-soluble stimulants before it is immersed in the sugar solution, hence the appearance and accumulation of the stimulants externally could be due merely to diffusion outwards of the internal supply. Diffusion does not account, however, for all the stimulants in the external fluid. In Fig. 5 it will be seen that the magnitude of the response of *Orobanche* at Rf 0·9 is much greater than the corresponding response in Fig. 2. The difference in the amounts of M_o which this suggests is accentuated by the fact that the data of Fig. 5 refer to a culture of 10 segments whereas the data of Fig. 2 refer to an extract of 60 segments. The evidence supports the view that the M_o stimulant is synthesized by the isolated root fragment.

The data carry one further implication. The quantity of M_o increases in the segment fluid with time, but the water-soluble stimulant does not. Indeed, there is no evidence that the water-soluble stimulant appears in the sugar solution. It is clear, however, that this material is a more prominent constituent than M_o in the tissue before the fragments are immersed in the sugar solution. It is suggested therefore that the failure of the water-soluble stimulant

to appear externally is due to its conversion within the tissue into an ether-soluble stimulant which then passes out into the sugar solution. Since *Orobanche* seeds reveal the presence of the water-soluble stimulant it may be further suggested that it is in fact a precursor of M_o.

ACKNOWLEDGEMENTS

I am indebted to Professor R. Brown, F.R.S., and his colleagues for putting their results at my disposal. The modified assay technique was suggested by and developed in collaboration with A. D. Greenwood, and it gives me great pleasure to record my gratitude to him for his valuable help and advice.

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Radioactive Oxygen in the Study of Gas Transport down the Root of *Vicia faba*

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SUMMARY

The movement of labelled oxygen down the main root of *Vicia faba* seedlings was studied. The rise of radioactivity in the roots at 15 and 18 cm. below the cotyledons follows the same kinetics as the transport of radioactive gas down a thin polythene tube open at one end. It is therefore consistent with diffusion of oxygen through continuous gas spaces in the roots. Loss of oxygen to the surrounding tissue is small in the upper parts of the roots. The volume of the gas spaces was estimated from the equilibrium radioactivity and compared with data obtained by filling the spaces with paraffin oil.

INTRODUCTION

In the course of some radiobiological experiments it became important to know whether continuous longitudinal gas spaces existed in the main root of *Vicia faba*, the broad bean.

Some evidence is available for the presence of intercellular spaces in root tissue. Hunter (1915) suggested that *Vicia faba* plants grown in aerated potting soil have gas spaces in the cortex of the root, which are joined to the gas spaces in the stems. He also observed intercellular gas spaces in the youngest tissues, i.e. right down to the tip of the root. The cotyledons have 'continuous intercellular gas spaces capable of bringing about a most active exchange of gases'. Norris (1913) observed that gas spaces were invariably present in the roots of *Zea mays* when cultured in water, but these were found to be reduced when the roots were grown in porous soil. The extent of the gas spaces depended on the culture conditions and on the availability of air to the cotyledons. However, we have been unable to find any work demonstrating the continuity of these gas spaces or the nature of the material occupying them. R. Brown (1947) has demonstrated transport of gases in the root of *Cucurbita pepo* and believes this to be an active translocation of gases in solution.

We have studied the diffusion of air labelled with the short-lived radioisotope O^{15}, in *Vicia faba* roots.

MATERIALS AND METHODS

Culture of bean roots. All experiments were carried out on roots of the broad bean *Vicia faba* (Sutton's Prolific Long-pod, 1957 crop). The dry beans were washed in soap, rinsed thoroughly in cold tap water, and finally with distilled water. They were germinated in distilled water at room temperature. The

distilled water ran through the germinating trough at a speed such that one complete change was effected every 3–4 hours, ensuring sufficient aeration. Seedlings showing a radicle of 3–5 mm. in length were then planted in moist horticultural vermiculite, where they were kept until their roots were 4–7 cm.

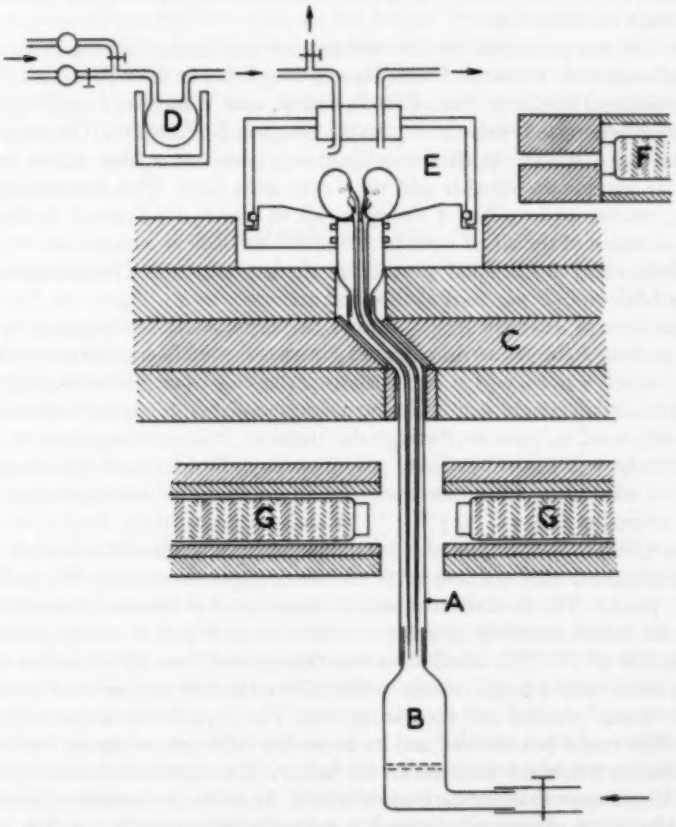

FIG. 1. Experimental arrangement used for O^{15} measurements. A, plastic tube; B, glass attachment with sintered filter; C, lead shielding; D, re-entrant ionization chamber; E, belljar; F, monitor counter; G, scintillation counters in coincidence.

long. The seedlings were removed carefully, the shoots severed at the cotyledons, the cotyledons freed of their seedcoat and transferred to the culture tank. The seedlings then remained in the dark, the roots being immersed in continuously aerated tap water at 19° C. The lateral roots as well as shoots were clipped daily. The main root grows fairly straight at a rate of about 2 cm. a day. The roots were cultured in these conditions up to the time of the experiment, when their average length was 22–25 cm.

Radioactivity and counting techniques. O^{15} has a half-life of 2 min. It is a pure positron emitter. The positrons give rise to annihilation radiation of two gamma quanta of 511 KeV, emitted in opposite directions. Two counters arranged in a coincidence circuit give good spatial resolution and low background. We used the counting system described by Dyson (in preparation) with some modifications.

The O^{15} was produced by the cyclotron of the Medical Research Council Radiotherapeutic Research Unit (Dyson, Hugh-Jones, Newbery, and West, 1958; Gallop, Vonberg, Post, Powell, Sharp, and Waterton, 1957). 15 MeV deuterons were used with air to give the reaction $N^{14}(d,n)O^{15}$. The air which had been exposed to the deuteron beam was passed first over heated cupric oxide or manganese dioxide and then over soda lime. This removed $C^{11}O$, $C^{11}O_2$, ozone, and oxides of nitrogen, all of which are formed during the bombardment of the air. A trace of A^{41} with a half-life of 2 hours was the only contaminant in the labelled air used in the experiments. This amounted to only a fraction of a per cent of the total activity.

Experimental assembly. Fig. 1 shows the experimental arrangement. The bean seedlings are shown in position. Up to ten roots were immersed in tap water, which was contained in the plastic tube A of 1 cm. diameter. The tube ended in a glass attachment B, having a sintered glass filter at the bottom. The filter was used to pass air through the water in order to oxygenate it. The plastic tube was bent in order to pass it through 8·9 cm. lead shielding C. A batch of nine roots left in the assembly for 20 hrs. with continuous aeration grew normally.

The O^{15}-labelled air passed through a re-entrant ionization chamber D. It could be mixed with inactive air to the desired specific activity. We used 20–60μC. per ml. The ionization chamber D was kept at a distance of about 15 m. from the actual assembly in order to reduce the number of counts due to the supply line of O^{15}. The labelled air was then passed through the belljar E and piped away into a large-volume baffle where the O^{15} was allowed to decay before it was released into the atmosphere. The appearance of the activity in the belljar could be observed and its constancy followed, using the collimated scintillation counter F directed at the belljar. The scintillation counters G in coincidence were shielded by lead cylinders. In order to obtain the maximum coincidence counting rate, counters G were placed as near as possible to the roots.

Experimental procedure. Spurious coincidence counts due to scattering can be excluded by counting only quanta with energies higher than half that of the annihilation radiation. The equipment was accordingly adjusted using the 320 KeV gamma rays from Cr^{51}. A check was made that spurious coincidences due to scattering were negligible.

The plastic tube (without the seedlings) was filled with water and active gas admitted to the belljar for some 20 minutes. The coincidence counting rate remained zero. This showed the absence of gas leaks and also that there was no movement of active gas 15 cm. down the water column.

A group of beans was inserted into the bean vessel and the surrounding tap water aerated gently. The cyclotron beam was switched on and the air-flow through the target chamber started so as to build up a steady activity of gas before starting the experiment. The aeration of the water around the roots was then stopped. Pen recorders were started, synchronization marks made, and active air allowed to flow through the belljar. Recordings were continued for 10 minutes or more, readings of monitor counters were noted occasionally, and the belljar flushed out with inactive air. Aeration of the roots was recommenced. When the activity had died to a sufficiently low level, the procedure was repeated. Periodically during the experiments, checks were made on the balance of the subtractor unit by introducing a delay into one arm of the coincidence unit.

EXPERIMENTAL RESULTS

As a measure of the taper and the uniformity of the bean roots used, we measured the weight of 20 roots cut into segments of 3 cm. (see Table I) as well as the weight of 10 segments 2 cm. in length between 14 and 16 cm. below the cotyledons. The mean weight of such a 2-cm. segment was 0·0347 g. with a standard deviation of 0·01 g.

The density and the volume of the gas spaces in root segments 1 cm. or 2 cm. long were measured, using Archimedes' principle. The method is similar to the one used by Nius (1931). He employed turpentine to measure gas spaces in green leaves because it did not mix with water. We used as immersing fluid paraffin with a density of 0·875 g. per ml. at 25° C. This method was chosen because it measures the volume of undissolved gas contained in a root segment irrespective of mechanical damage inflicted during measurement, and avoids the removal of gases dissolved in tissue fluids. Root segments 1–2 cm. in length taken at about 15 cm. from the cotyledon were cut with a scalpel under paraffin oil, and suspended from a balance by means of a thin wire weighed previously in the same position dipping into the oil. The bean root segments still in the oil were cooled in an ice bath and exposed to a vacuum of about 20 mm. Hg until no more gas bubbles could be withdrawn. The segments were weighed again and the increase in weight was taken to be due to displacement of gas by oil. The root segments were then removed from the oil, wiped carefully with filter-paper, and weighed in air. The gas spaces were found to have a volume of $6{\cdot}8 \pm 1{\cdot}2$ per cent. of the volume of the root segment.

Some measurements were made of the rate of oxygen consumption of roots grown under our conditions. They were put in a vessel containing air saturated with water vapour. The oxygen content of small samples of the air was estimated at intervals, using a Hersch cell (Hersch, 1954). A value of about 0·4 μl. per mg. wet weight per hour was obtained for the mean Q_{O_2} of root tissue at about 25° C.

From the recorder traces of the O^{15} experiments, the activity of the selected region of the bean roots, averaged over 10- or 30-second periods, was obtained as a function of the time after the O^{15} gas was admitted to the belljar. Figs. 2

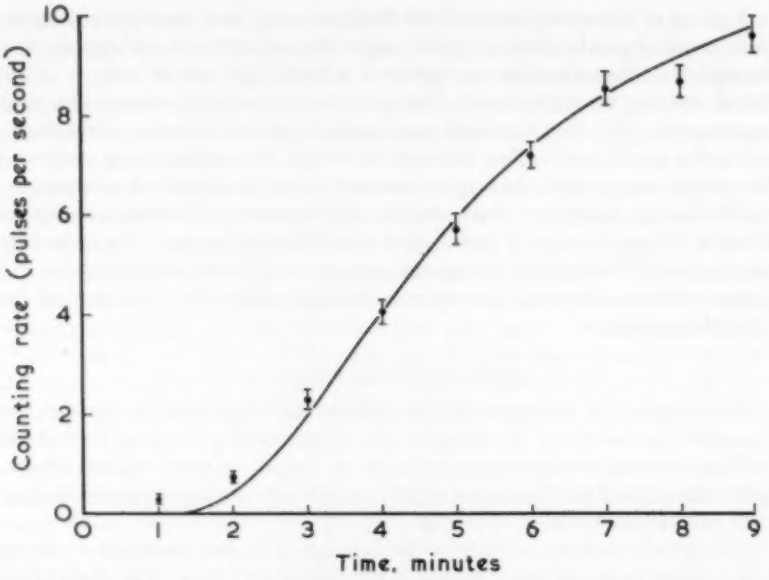

FIG. 2. Radioactivity of bean roots 15 cm. below the cotyledons. Ordinate: counting rate in pulses per sec. Abscissa: time in minutes after admitting labelled gas.

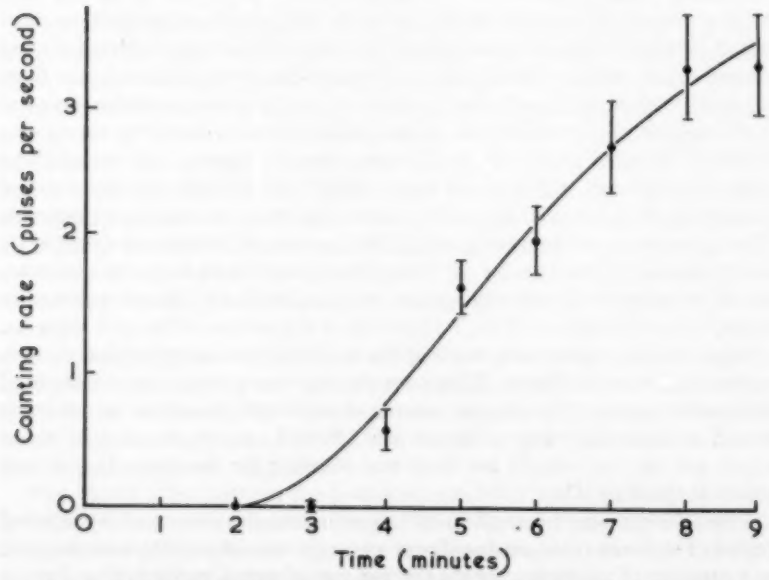

FIG. 3. Radioactivity of bean roots 18 cm. below the cotyledons. Ordinate: counting rate in pulses per sec. Abscissa: time in minutes after admitting labelled gas.

and 3 show the values for two typical runs obtained with a group of five beans, at distances of 15 and 18 cm. below the cotyledons respectively. The results obtained from similar experiments with an air-filled polythene tube of internal diameter 0·57 mm. sealed at the lower end and in the position of the bean roots are shown in Fig. 4. The errors indicated in Figs. 2, 3, and 4 are those

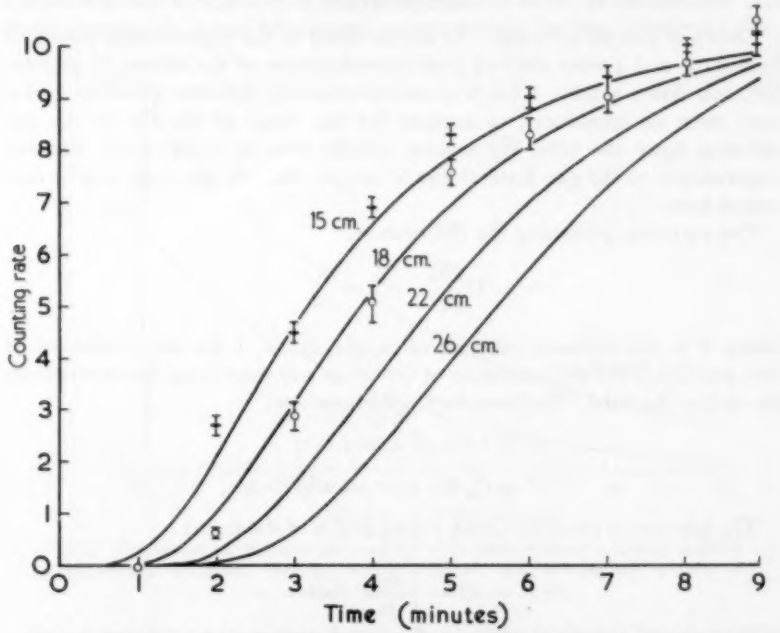

FIG. 4. Radioactivity of air-filled polythene tube at 15 and 18 cm., with theoretical curves for gaseous diffusion at 15, 18, 22, and 26 cm. Ordinate: counting rate in arbitrary units normalized at 10 min. Abscissa: time in minutes after admitting labelled gas.

due to counting-statistics alone. The curves are theoretical and will be discussed later. About a dozen repeats of these experiments were carried out with similar results. It can be seen that both in the bean roots and polythene tube the build-up of the radioactivity is of sigmoid form. The steady levels of activity reached after about 9 minutes in four experiments with bean roots and polythene tubes were about 9·6, 3·5, 20, and 30 counts per second respectively, the corresponding values of the specific activity of the gas in the belljar being 43, 42, 21, and 54μC. per ml. The decay of activity from its equilibrium value after removal of the active gas from the belljar was recorded for the roots and polythene tube. In each case the decay during the first 5 minutes was very much slower than the 2-minute radioactive half-life of O^{15}. Both the rates of appearance and disappearance of the activity in the bean root are consistent with the gaseous diffusion theory discussed later.

Estimates of the air volume in bean roots were then made by direct comparison of their steady state activities with that of a polythene tube. For two groups of five beans each, the volumes of root segments thus calculated to be occupied by labelled air were $1{\cdot}6 \pm 0{\cdot}2$ per cent. and $3{\cdot}6 \pm 0{\cdot}25$ per cent.

DISCUSSION

Theory of gaseous diffusion. The curves fitted to the experimental points of Figs. 2, 3, and 4 were derived from considerations of the theory of gaseous diffusion down a tube. This is a one-dimensional diffusion problem and a term must be introduced to account for the decay of the O^{15} in the gas diffusing down the tube. We assume initially that the walls of the tube are impermeable to the gas. Lateral loss of oxygen through the walls will be discussed later.

The equation governing the diffusion is:

$$D\frac{\partial^2 C}{\partial x^2} - \lambda C = \frac{\partial C}{\partial t}$$

where D is the diffusion constant of oxygen in air, λ the decay constant of O^{15}, and $C(x,t)$ the concentration of $O^{15}O^{16}$ at any time t and distance x from the end of the tube. The boundary conditions are:

$$C = 0 \text{ for all } x, \text{ at } t = 0$$

$$C = C_0 \text{ for } x = 0, \text{ and } t > 0.$$

The solution is given by Crank (1956) and is of the form:

$$C = \int_0^t \frac{\partial \phi}{\partial t} e^{-\lambda t}\, dt$$

where

$$\phi = C_0\left(1 - \frac{2}{\sqrt{\pi}} \int_0^u e^{-u^2}\, du\right) \tag{2}$$

$$u = \frac{x}{\sqrt{(4Dt)}}.$$

For all the calculations, the following values were used:

$$D = 0{\cdot}187 \text{ cm}^2 \text{ sec}^{-1}$$

$$\lambda = 0{\cdot}0577 \text{ sec}^{-1}$$

The evaluation of the integral in equation (2) was carried out graphically, using tables for the derivative of the error function given by Jahnke and Emde (1945).

Curves of C against t for four different values of x are shown in Fig. 4. The curves have been normalized to the value 10 at time $t = 10$ minutes. Fig. 5 shows the variation of concentration with x for $t = 4$ and 10 minutes, the

concentration being unity at $x = 0$. In the present case, the counting rate r at any time and point in the root should be given by:

$$r(x,t) = \alpha A \lambda C(x,t) \tag{3}$$

where α is a constant factor depending upon the geometry of the counting system and the scintillation crystal efficiencies. A is the cross-sectional area of the gas spaces. α was determined experimentally for the polythene tube.

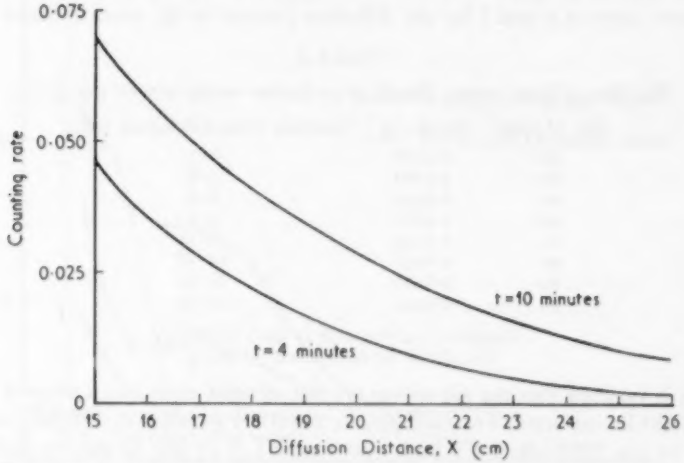

FIG. 5. Theoretical variation of radioactivity with distance down a tube of uniform cross section. Ordinate: counting rate in arbitrary units normalized to unity at $x = 0$. Abscissa: diffusion distance in cm.

By adjusting the parameters A and x in equation (3) it was found possible to fit the observed experimental points for the transport of oxygen down the roots and the polythene tube quite closely. Fitted theoretical curves are shown in Figs. 2, 3, and 4. It was found that the value of x required to obtain the best fitting curve was greater than the observed distance l from the base of the cotyledons to the point midway between the two counters. Thus, for a length of root $l = 15$ cm., x was about 21 cm. Other groups of beans showed similar behaviour.

A further check of equation (3) was carried out. Fig. 3 shows the rise of activity at $l = 18$ cm. A theoretical curve is fitted with $x = 24$ cm., and using the same value of α and A as in Fig. 2. The agreement of theory and experiment suggests that equation (3) is a valid description of the oxygen transport. It should be noted that the theory assumes the walls of the tube to be impermeable to oxygen.

A similar calculation for the diffusion of O^{15} through still water shows that no activity could appear at $l = 15$ cm. This is a consequence of the short half-life of the isotope.

Discrepancies between estimates of air volume. The results based on the steady-state activities of the two groups of roots showed larger differences than could be expected from the random variation in the weights of 2-cm. sections from different roots (Table I). This may reflect some anatomical differences such as developmental differences of the lateral root initials in the two groups of beans used. However, there were considerable discrepancies between the estimates based on density measurements (6·8 per cent.) and those derived from the O^{15} measurement (1·6 and 3·6 per cent.). The difference between x and l for the diffusion process in the roots explains this,

TABLE I

Weights of bean roots: Batch of 20 beans—mean length 25·46 cm.

No. of roots	Weight (g.)	Distance from cotyledons (cm.)
20	8·4568	0–3
20	3·7083	3–6
20	2·2444	6–9
20	1·4811	9–12
20	1·0249	12–15
20	0·8056	15–18
20	0·6966	18–21
20	0·5490	21–24

Weight of 14 root tips (1 cm.) 0·0883 g.
Weight of 20 cotyledons 73·261 g.

since it suggests that the gas spaces are not straight pipes but contorted ones as might be expected. For example, we observed an effective diffusion length $x = 21$ cm. $\pm 0{\cdot}6$ cm. for a bean root length $l = 15$ cm. It can be deduced from Fig. 5 that the specific activity at $t = 10$ minutes is higher by a factor 3·0 at 15 cm. than at 21 cm. Correspondingly, the best estimates of the gas spaces are increased to between 2·5 and 10 per cent., and to $10{\cdot}7 \pm 1{\cdot}5$ per cent. respectively. These values are now in adequate agreement with the estimates obtained from the density measurements. This difference in apparent diffusion length in the bean root and in the polythene tube may be due to several causes. There could be contortions leading to an increase in length of 40 per cent. These contortions could occur within a small length of the diffusion path, say the cotyledon, or might be distributed along the whole length of the path. Barriers such as a thin water layer or permeable membranes might have a similar effect. A further development of the O^{15} techniques should make it feasible to distinguish between these possibilities.

The air volumes in the bean root have been calculated from O^{15} activities by comparison with measurements on polythene tubes, and the following assumptions were made:

(*a*) Oxygen is transported down the tube by gaseous diffusion.
(*b*) The lining of the gas spaces is impermeable to oxygen.
(*c*) The gas contained in the spaces has the same oxygen content as air.

If assumption (*b*) is no longer made, it is possible to obtain a lower limit for the diameter of a cylindrical gas tube along which oxygen diffuses. Oxygen is

likely to be lost laterally from the tube into the root tissue by diffusion. Even if the metabolism of the tissue is neglected and the diffusion constant for O_2 is taken to be half that through water, i.e. 1×10^{-5} cm.^{2} sec^{-1} through the root tissue, the lateral flux from a 15-cm. length of tube becomes equal to the downward flux at 15 cm. for tube diameters of about 150μ. Tubes of smaller diameter would be depleted of oxygen over a much shorter length. On the

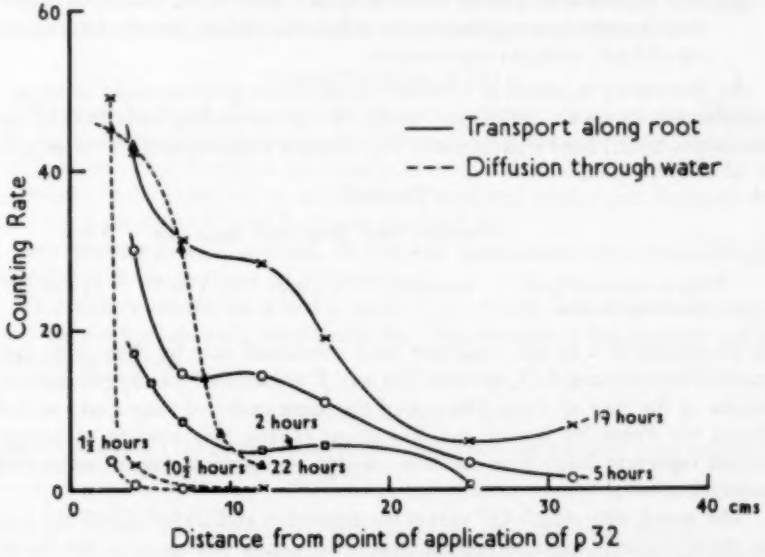

FIG. 6. Movement of P^{32} along a bean root, and through a tube containing distilled water (Gray, unpublished). Ordinate: counting rate. Abscissa: distance from point of application of P^{32}.

other hand, for tubes having a diameter great compared with 150μ, the lateral loss becomes less important. Our estimate of the cross section A of the diffusion space would be too large if such lateral loss occurs at the place of measurement, and conversely too small if the loss occurs above the place of measurement. On the basis of our results, it seems likely that lateral diffusion is small. This suggests, therefore, either that the oxygen permeability of the material lining the gas spaces is very much lower than that of water, or else that the diameter of the spaces is at least 150μ if they are of cylindrical cross section. The ratio of volume to surface is smaller for any other shape, thus leading to even larger diameters. In longitudinal, as well as in transverse sections of our roots, we have never seen spaces approaching 150μ in diameter. If metabolism of oxygen is taken into account, the loss of oxygen from the gas spaces becomes considerably greater. This only emphasizes the above argument of the need for even larger spaces or for an even lower permeability of the walls to oxygen.

The impermeability of the lining of the spaces containing gas is therefore strongly supported by three facts:

(*a*) No cylindrical spaces of 150μ or more in diameter can be seen in bean roots cultured under our conditions.

(*b*) The O^{15} is not freely available for metabolism by the upper part of the root.

(*c*) The depletion of activity between 15 cm. and 18 cm. below the cotyledons is entirely accounted for by radioactive decay, provided the spaces are of fairly uniform cross section.

An interesting question is whether the diffusion process might serve as a supplementary means of oxygen supply to the extending cells behind the meristem, which have a particularly high oxygen consumption. If the oxygen

TABLE II

	Polythene tube O^{15}	Bean root O^{15}	Bean root P^{32}	Water P^{32}
Time to reach 20% of final equilibrium	2·0 min.	2·9 min.	*c.* 1 hr.	> 24 hrs.

in the spaces of a 25-cm. long root were consumed only by these cells they could obtain about 9μl. O_2 per hour this way. Provided that the oxygen requirements of the root of *Vicia faba* are of the same order of magnitude as that found for *Pisum* by Brown and Broadbent (1950), this amount of oxygen would represent more than the total oxygen requirement for the extending zone.

The speed with which O^{15} enters the cotyledon and travels down the root is extraordinarily rapid in comparison with active transport in the liquid phase as observed by L. H. Gray. He studied the movement of P^{32} down bean roots. His data have not been published before, and he very kindly allowed us to quote his experiments in this communication.

A long root without shoot or laterals was selected, and a small hole scooped out of one of the cotyledons. Into this hole a drop of solution containing P^{32} (probably as phosphate) was introduced. When not being measured, the root was stored in a vessel containing tap water. From time to time the activity of about 1 cm. of root was examined by means of a beta-ray counter. For this purpose a lead tube was slipped over the root and the activity measured through a window in the tube. In order to compare the movement of phosphorus down the root with physical diffusion under similar circumstances, a glass tube was drawn down so as to have approximately the same diameter and taper as the root. This was filled with water and a small volume of P^{32} solution was introduced at the wide end of this tube in a position approximately corresponding to the cotyledon of the bean. Beta-ray counts were made with the same lead shielding tube and window as was used for measurements on the root. The observations are shown in Fig. 6, in which the distribution of the activity is plotted for different times both in the bean root and

the column of water. A comparison of these results with our own is shown in Table II.

It can be seen that the processes of gaseous diffusion, active transport in the liquid phase and diffusion through water involve time scales differing in orders of magnitude. The rapidity with which O^{15} travels through the bean cotyledon and root is quite comparable with the ventilation of the polythene tube. This, we feel, is very strong evidence for the existence of a system of continuous spaces in the plant facilitating rapid gas movements.

ACKNOWLEDGEMENTS

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We are most grateful to Dr. L. H. Gray for permission to quote his hitherto unpublished observations on P^{32} transport.

Our sincere thanks are also due to Dr. J. L. Harley for valuable criticisms and for discussions with members of the Departments of Agriculture and of Botany at Oxford.

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A New Micro-osmometer

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SUMMARY

This apparatus measures the rate of distillation at room temperature between the unknown solution and a known standard solution under carefully defined conditions. It is calibrated using solutions of known osmotic pressure. It is therefore an empirical vapour-pressure osmometer. The sample (0·06 ml.) is placed in the capsule *C* (Fig. 1) and the standard solution contained in the pipette *P*, where it is held at a fixed position due to surface tension at the jet. The upper meniscus is located on the eye-piece scale of the microscope *M*. The capillary tube above the meniscus and the space inside the screw plunger *S* are air filled. By turning the screw *S* the standard solution can be extruded to hang as a drop from the tip of the jet. After a measured time (20 min.) the drop is again drawn into the pipette to the fixed position at which it comes to rest automatically, and the change in volume, due to condensation or evaporation, measured on the microscope scale.

If the sample has a surface tension not much lower than that of water, the osmometer can be used in the reverse way, the sample being drawn into the pipette and the standard in the capsule. Used in this way only 0·001 ml. of the sample is needed.

A separate calibration curve is required for each standard (Fig. 2) and each standard has a range of about 9 atm. ($\Delta = 0{\cdot}75^\circ$ C.). The standard deviation of single determinations is $\pm 0{\cdot}06$ atm. ($\Delta = 0{\cdot}005^\circ$ C.). This is uniform over the range investigated (0–28 atm. or $\Delta = 2{\cdot}3^\circ$ C.).

Satisfactory comparisons between the osmometer and the cryoscopic method have been obtained with samples of plant sap (Table I).

The osmometer has the advantage of needing only a small volume of solution which can be viscous, opaque, and need not be free from cell debris, &c. It operates at physiological temperatures and needs no very stringent temperature control. It is simple to construct and manipulate and occupies less than 1 hour per determination of which only 15 minutes is taken up in operation.

INTRODUCTION

THE problem of measuring the osmotic pressure of fluids where the total volume available is 0·1 ml. or less is frequently encountered in biological work. Several micro-methods are available based on cryoscopy (Ramsay and Brown, 1955) or vapour pressure, either directly (Barger, 1904; Halket, 1913; Ursprung and Blum, 1930) or indirectly by measuring temperature differences arising in evaporating drops (Baldes and Johnson, 1939; van Andel, 1952). A disadvantage with many of these methods is that either they need rather elaborate equipment or they are tedious to carry out. The apparatus described in this paper is simple to use, easy to construct, and requires little more equipment than is normally found in the laboratory.

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The principle of the method may be understood with reference to Fig. 1. Capillary tubing is drawn out to a jet at its lower end to form the pipette *P* and bears a ground-glass cone *G*. At its upper end it is attached to the screw plunger device *S*, the cavity of which is air filled. Solution of known osmotic pressure (O.P.) is drawn into the pipette by immersing the tip of the jet and unscrewing *S* until the meniscus approaches the line *I*. Once inside the tube the thread of solution is held in a fixed position at its lower end by virtue of surface tension at the orifice of the jet. About 0·1 ml. of the sample is placed in the capsule *C* which is then fitted on to the cone *G*. The osmometer is now fixed vertically in a glass-sided water bath and the exact position of the meniscus in the pipette, with the fluid at its automatic setting position, located on the eye-piece scale of the microscope *M*. By turning *S* the solution in the pipette is now extruded so that it hangs as a drop from the tip of the jet. In this position either the drop will evaporate or water vapour will condense on it according to the difference in activities between the water in the drop and that in the capsule. The change in volume of the drop occurring during a standard time is measured by drawing the drop back into the tube to its setting position and locating the meniscus once again on the eye-piece scale. The method is empirical, the apparatus being calibrated by putting solutions of known O.P. in the capsule and constructing a calibration curve from the data so obtained.

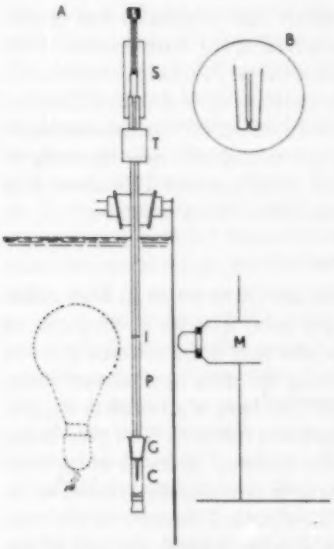

FIG. 1. (A). The osmometer in position in the water bath. *C*, capsule; *G*, ground glass cone; *I*, reference line; *M*, travelling microscope; *P*, pipette; *S*, screw plunger device; *T*, pressure tubing.
(B) Magnified view of jet.

With an osmometer of the dimensions described in this paper, at least 0·06 ml. of the sample solution are needed to wet the inside of the capsule uniformly. The volume of the sample needed could undoubtedly be reduced by using a smaller capsule, but this possibility has not been investigated. Where only very small volumes of sap are available the osmometer can be used in the reverse way: standard in the capsule and sample in the pipette. This requires only 0·001 ml. of the sample. Unfortunately the surface tension of many biological fluids is low enough to prevent their hanging properly from the jet, but this method has been successfully applied to xylem and phloem saps. If desired, the instrument could give an absolute measure of O.P. by allowing the standard to come to equilibrium with the sample in the capsule. If the initial and final volumes of the standard were known, its O.P. at equilibrium and hence that of the sample could be computed. No investigation has been

made of the time taken to come to equilibrium and it is doubtful whether this would have any advantage over the much quicker dynamic method described above.

In addition to simplicity and ease of operation this osmometer has certain advantages. The activity of the sample is measured at the temperature of the water bath which can be adjusted to any desired value. Freezing, which could lead to precipitation or gelation in certain circumstances, is avoided. Also the presence of solid matter, cell debris, &c., should not affect the measurement if the sample is placed in the capsule. Again, determinations may be made on strongly coloured or even opaque fluids which could present difficulties with micro-cryoscopic methods involving the observation of ice crystals.

DETAILS OF CONSTRUCTION

(*a*) *The pipette.* This is 20 cm. long and made from 0·1 mm. bore soda-glass tubing with an external diameter of 4·5 mm. The jet is prepared as follows. A dilation of the bore a few cm. from one end of the selected piece of tubing is made by sealing one end and rotating the tube in a narrow flame whilst air pressure is applied at the other end. The bore is allowed to expand to 2 or 3 times its normal diameter over a length of a few mm. The tube being heated at the dilation, it is pulled out until the external diameter at its most constricted is between 0·5 and 1 mm. The tube is now cleanly severed at its narrowest point. In this way a narrowing of the external diameter of the tube with little corresponding change in bore is achieved. Finally the end of the jet is gently heated in a flame no more than a few mm.^{3} in size. This smooths the edges and produces a slight constriction of bore at the tip as shown in Fig. 1B. It is this constriction which holds the solution in the pipette in a fixed position.

The commercially produced ground-glass cone *G* was threaded over the jet and sealed on to the capillary in the position shown with epoxy resin cement ('Araldite'). Some 8 or 10 cm. above the jet a reference line *I* is painted on the glass to indicate the approximate level to which liquid is drawn into the pipette.

The screw device *S* is made from a block of perspex, tapped to take a 1 B.A. steel screw and having a short length of thick-walled tubing sealed in with 'Araldite'. The threads of the screw are smeared with grease so that when attached to the top of the pipette by the pressure tubing *T* the whole device is air-tight.

A little below the screw plunger the pipette passes through a 24 mm. ground-glass cone into which it is rigidly fixed by a cork (rubber is too pliable). A 24 mm. socket is attached to the water bath a little above the water level and this holds the osmometer firmly in position in the water bath as shown in Fig. 1A.

Before use, the outside of the jet and the internal walls of the pipette are made water repellent by washing in 'Repelcote' (2 per cent. dimethyldichlorosilane in carbon tetrachloride) according to the makers' instructions.

(*b*) ***The capsule.*** This consists of a length of glass tubing 2·5 cm. long by 7 mm. internal diameter and bearing a ground-glass socket to fit the cone *G*. The inner surface of this capsule is ground with coarse carborundum so that the walls will hold an appreciable film of liquid when wetted. Lining the walls with filter-paper is to be avoided owing to its considerable adsorption of ions. The lower end of the capsule is closed by a rubber bung.

(*c*) ***The water bath.*** This is an aquarium tank 60×30×30 cm., lagged on three sides with glass wool. Temperature is controlled by a mercury-in-glass contact thermometer combined with a 100-watt immersion heater and centrifugal pump stirrer. No variation in temperature could be detected on a thermometer graduated in tenths of a degree. The water bath was situated in a constant-temperature room (25°±1° C.) and was operated at the same temperature as the room. This can be done, since without control a water bath in a constant-temperature room will reach a steady temperature 1 or 2° C. below that of the room. The advantage of keeping the bath at the same temperature as the room is that no time need be allowed for temperature equilibrization on immersing the osmometer in the bath. (For further information on temperature requirements see Appendix I.)

(*d*) ***The travelling microscope.*** This permits lateral as well as vertical movement. It is fitted with a 2-in. objective and has an eye-piece micrometer on which all the readings are taken; the vertical and lateral movements are merely used for positioning the microscope. The osmometer is illuminated by a 100-watt bulb fixed outside the bath and shining through the side panel a little behind the capillary (see Fig. 1). With careful adjustment of the position of the light and focus, the meniscus appears as a clear straight boundary between light and dark areas of the field.

It is important that there should be no relative movement between the microscope and the osmometer during operation. Firm emplacement is essential.

With its lateral travel, one microscope might serve a row of several osmometers operating simultaneously.

MODE OF OPERATION

Solution can be introduced conveniently into the pipette from 1 mm. bore siliconed quill tubing. A length of this is dipped into the solution so that a little is held by surface tension in the terminal few mm. The pipette is clamped in a vertical position jet downwards (a 24 mm. socket held in a clamp stand is useful for this), and the quill tubing pushed over the jet from below so that the tip is immersed. Solution is then drawn into the pipette to a position about 3 mm. below the line *I* and the quill tube withdrawn. With the bung in position (its exact degree of insertion is not important) approx. 0·1 ml. of solution is run into the capsule from a micro-pipette. Having luted the cone *G* with vaseline, the capsule is now put into position. Marks are made on the pipette and capsule so that they are always put together in the same radial position and the jet kept in the same position relative to the walls

of the capsule. In putting the osmometer together the pressure in the capsule is often raised slightly and this causes the solution in the pipette to rise. By adjusting the screw plunger the solution can be driven back to its setting position.

The osmometer is now removed from its stand, held in a horizontal position and rotated until the walls of the capsule are thoroughly wetted. The osmometer is then placed in position in the water bath and the meniscus brought into focus. At this point the automatic setting should be tested: by screwing the plunger down a little the solution will begin to run out of the pipette, the plunger is now immediately turned back and the solution made to rise again until it stops automatically, when the meniscus at the lower end is held in the orifice of the jet. This operation may be watched through the microscope and on repetition the meniscus should come to rest each time at the same reading on the micrometer eye-piece. If it shows irregular setting the surface of the jet or the inside of the pipette is dirty. This may be rectified by washing with acetone and if this fails it is necessary to wash the pipette with alcoholic potash and reapply silicone.

The solution is now extruded by regulating the screw plunger until the upper meniscus reaches a point 2 or 3 mm. above the end of the jet. Almost all the solution is now hanging as a drop from the tip of the jet. No difficulty is experienced in observing the jet through the walls of the capsule even with strongly coloured solutions. The apparatus is left to settle for 10 minutes, after which the drop is withdrawn, and if the rate of rise of solution in the pipette is not too rapid it will stop automatically at its setting position. The vertical position of the microscope is now adjusted so that the meniscus lies exactly at the midpoint of the scale, thus allowing the maximum possible excursion in either direction. The setting is again tested as described above and the solution extruded for exactly 20 min. timed on a stop-watch. Two 20-minute readings are taken, if necessary the microscope being adjusted again to make the midpoint of the scale level with the meniscus before the second reading. Each determination is thus based on the mean change in volume of the drop during the 40-minute period immediately following the 10 minutes allowed for settling.

When plant sap is being placed in the capsule it is essential to wash the capsule in chromic acid after each determination or the sap will not flow easily over the walls and wetting will not be uniform. For this reason also the capsule is kept in chromic acid when not in use. To ensure that no grease from the socket is carried to the walls of the capsule, the lower end is closed by a bung rather than sealed so that it can always be washed through from the lower end.

CALIBRATION

Sodium chloride solutions were used for calibration and form the standards against which samples are measured. This salt has the advantage that its solutions keep well and its osmotic properties have been thoroughly worked out. A table of molalities and corresponding osmotic pressures calculated from

osmotic coefficients (Robinson and Stokes, 1955) is given in Appendix II. For the greatest accuracy it was found preferable to keep the difference between the O.P. of the solutions in the drop and the capsule less than about 5 atm. Standards of 0·1 M. (4·57 atm.), 0·3 M. (13·55 atm.), and 0·5 M. (22·56 atm.) were used, the first for determining the O.P. of samples from 0 to 9 atm., the second from 9 to 18 atm., and the third from 18 to 27 atm. Thus the approximate value of the O.P. of the sample should be known or ascertained in a preliminary determination.

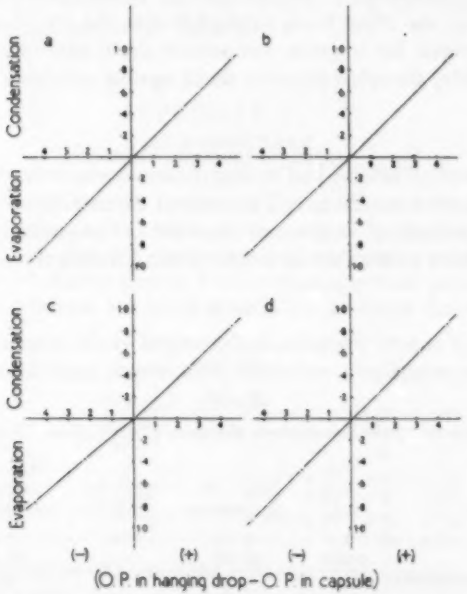

FIG. 2. Calibration curves. *a*, *b*, and *c* using 0·1 M., 0·3 M., and 0·5 M. sodium chloride solutions respectively as standards in the pipette. *d*, using 0·1 M. sodium chloride as standard in the capsule. Ordinates: Mean rates of evaporation by, or condensation on to, the hanging drop in scale divs. per 10 min.

Abscissae: difference in osmotic pressure between the standard and the unknown solutions in atmospheres.

There is no single calibration curve for a given osmometer, for the rate of vapour transfer per unit difference in O.P. between drop and capsule declines slightly the higher the absolute level of the O.P. Thus a separate calibration curve must be obtained for each standard and for either of the modes of operation, i.e. standard in the pipette or in the capsule. Calibration curves for the three standards (unknowns in the capsule) are shown in Fig. 2 *a*, *b*, and *c*, whilst *d* shows a calibration for 0·1 M. NaCl (unknowns in the pipette). Each point on the graphs is the mean of at least two determinations.

It will be seen that using 0·1 M. sodium chloride as standard a straight line

is obtained which is slightly displaced from the origin, i.e. there is a small rate of evaporation from a drop of O.P. 4·57 atm. with a solution of 4·57 atm. in the capsule.

With 0·3 M. standard the slopes on either side of the origin are not quite the same and this break in the calibration is somewhat accentuated using a 0·5 M. standard. These relationships are linear on either side of the origin and the differences in slope can be accounted for by the changes in the O.P. of the drops which result from the change in volume occurring during the operation of the osmometer. This drift in O.P. is more marked the higher the O.P. of the drop, the effect being negligible with the 0·1 M. standard.

Once determined for a given osmometer these calibrations seem to be remarkably stable, though a periodic check against solutions of known O.P. is advisable.

PERFORMANCE

In the course of calibrating and testing the osmometer twenty-four duplicate determinations were carried out. The error of determination has been calculated from an analysis of variance of these data. The standard deviation was ±0·06 atm., which means that for single determinations the confidence limits

TABLE I

Comparison of osmotic pressures as determined by the osmometer and those determined cryoscopically, calculated from osmotic coefficients or measured directly

Each determination using the osmometer is the mean of two values. Temperature 24·9° C.

Sample	Vol. used ml.	O.P. by osmometer	O.P. by Δ F.P.	O.P. from osmotic coefficient	Direct measurement*
0·3 M. Sucrose	0·1	7·50	—	7·53	7·48
1·0 M. Sucrose	0·001	26·56	—	26·45	26·80
0·1 M. Potassium nitrate	0·1	4·36	—	4·44	—
	0·001	0·89			
Tomato xylem exudate	0·1	0·89	0·90	—	—
Beet leaf sap	0·1	7·42	7·43	—	—
Beet root sap	0·1	12·80	12·85	—	—

* Adjusted from Berkeley and Hartley (1916).

($p = 0·05$) were ±0·12 atm. For the means of duplicate determinations the confidence limits were ±0·088 atm. Superficial examination of the data indicates that the errors were much the same over the whole range of determinations, so that whilst the coefficient of variation was 6 per cent. for single determinations of a solution of O.P. 1 atm. it was only 0·3 per cent. for a 20 atm. solution.

Each of these determinations was based on two consecutive 20-minute readings. The excursion of the meniscus is cumulative and presumably the error would be reduced if longer periods were chosen, indeed it might be possible to measure the O.P. of very dilute solutions with readings taken over

some hours. Conversely some accuracy could be sacrificed for speed, but these points have not been investigated.

The osmometer has been tested against sucrose and potassium nitrate solutions and against three different kinds of plant sap; that squeezed from the leaves and roots of beet after freezing and thawing and the exudate from the xylem of detopped tomato plants. The measured values of O.P. of the solutions were compared with values calculated from osmotic coefficients or direct measurements if available. The plant saps were checked by depression of freezing-point determination on the bulk sample using the method of Johlin (1931). The results are set out in Table I.

APPENDIX I

Possible Sources of Error

(*a*) *Standardization of time for initial settling and interval between readings.* The data for consecutive 10 min. readings taken withou tpreliminary settling are given in Table II. They show that the first reading is sometimes markedly different from the others, and thereafter there is a steady declining trend particularly where more concentrated solutions are being used. Thus an initial ten-minute period

TABLE II

O.P. in pipette atm.	O.P. in capsule atm.	Time intervals of 10 min. 1	2	3	4
4·57	0	0·75	0·88	0·90	0·89
4·57	9·06	0·88	0·81	0·81	0·80
13·55	18·05	0·89	0·86	0·86	0·84
22·57	27·14	0·91	0·89	0·87	0·82

Figures in the body of the table show the excursion of the meniscus in divs. of the eye-piece scale per 10 min.

should be allowed for the osmometer to settle; during this the drop must be extruded in the normal way. There may also be a 'jet error' due to small quantities of fluid which fail to clear from the surface of the end of the jet when the drop is drawn into the pipette. This and the steady decline in the readings makes it essential to use a standard period for taking readings both in calibration and subsequent use of the osmometer.

(*b*) *Volume of fluid in the capsule.* Replicate determinations using 0·1 M. sodium chloride solution in the pipette and various volumes of 0·2 M. solution in the capsule are shown in Table III.

Evidently there is no difference between 0·06 and 0·15 ml., but an erroneous reading was obtained using 0·05 ml. This was because 0·05 ml. was insufficient to wet the inside of the capsule and form a meniscus covering the rubber bung. Above that limit the volume can be varied widely without affecting the reliability of the readings.

(*c*) *Volume of the hanging drop.* Normally the meniscus in the pipette was initially about 3 mm. below the line which was itself 8 cm. above the jet. In the following experiments the initial position was varied between wide limits. 0·1 M. and 0·2 M. sodium chloride solutions were used throughout. Results are shown in Table IV.

Clearly the smaller the volume of the drop (proportional to the length of pipette occupied) the smaller was the reading obtained. It is thus necessary to standardize

TABLE III

Ml. of 0·2 M. NaCl in the capsule	Excursion in divs./10 min.
0·05	0·977
0·06	0·885
0·07	0·898
0·10	0·905
0·15	0·900
Least significant difference .	. 0·035

TABLE IV

Distance below the line (mm.)	Total length from jet to meniscus (mm.)	Excursion in divs./10 min.
1	79	0·905
3	77	0·905
10	70	0·843
20	60	0·800

TABLE V

The effect of changing the temperature of the water bath (the room temperature remaining at 25° C.)

Temperature of bath	Excursion in divs./10 min.	Equivalent in atm.
22·7° C.	0·874 0·854	8·84
24·9° C.	0·900 0·905*	9·06
Falling 24·85–24·7° C.	0·898	9·02

* Mean of calibration data.

the volume used in the pipette, but this need not be done very accurately, for the data in Table IV indicate that a difference of 1 mm. in the initial position of the meniscus would lead to an error of only 0·025 atm.

(*d*) *Temperature control.* Temperature control of the bath was better than ±0·01° C. using the equipment described above, and throughout the development of the osmometer a temperature of 24·9° C. was maintained within these limits. However, to investigate the effect of temperature fluctuations on the operation of the osmometer the temperature control was discontinued and the bath allowed to equilibrate with the room temperature (25°±1° C.). In fact it became steady at 22·7° C. and with continuous stirring remained constant to ±0·05° C. over short

periods. Two determinations using 0·1 M. and 0·2 M. sodium chloride solutions were made at this lower temperature. The temperature of the bath was then raised and held thermostatically at 24·9° C. and a third determination made. Finally the heating element was switched off and a determination made on a falling temperature. The results are shown in Table V.

It appears that a difference of 2·2° C. altered the measured osmotic pressure by about 0·22 atm. This is accountable as due to the effect of temperature on the O.P. of the solutions and on the rate of diffusion of vapour between the drop and the walls of the capsule. A slowly falling temperature had little effect on the determination. In view of these results it seems likely that very precise control of temperature is unnecessary; indeed if the control of the room itself is good, mere stirring of the water bath might well be adequate.

TABLE VI

The effect of changing the room temperature, the temperature of the bath remaining at 24·9° C.

Room temperature ° C.	Period of temp. equilib'n minutes	Molality of solution in pipette	Molality of solution in capsule	Excursion in scale div./10 min.
21	15	0·1	0·2	0·940
21	30	0·1	0·2	0·945
21	15	0·5	0·4	0·875
25	24	0·1	0·2	0·930
25	15	0·5	0·4	0·865
25	0	0·1	0·2	0·905

In the absence of a constant-temperature room a slightly modified procedure is necessary. The bath would be controlled some 4 or 5° C. above room temperature. It is necessary therefore to allow the osmometer on immersion to attain the temperature of the bath *before* extruding the drop for the usual 10-minute settling period. To test the efficacy of this procedure the temperature control of the room was discontinued but control on the bath maintained. The excursion using standard sodium chloride solutions was measured using various periods for temperature equilibrization. The room was then returned to its controlled temperature and the procedure repeated. The results are set out in Table VI.

It is evident that provided 15 minutes or more are allowed for the osmometer to attain the temperature of the bath, the temperature of the room has little effect on the reading obtained. The last line of the table shows that even when the room and bath were at the same temperature a period of equilibrization altered the reading appreciably. This emphasizes that the procedure used in calibrating the instrument must be followed exactly in the determination of unknown solutions.

APPENDIX II

Osmotic pressure of sodium chloride solutions at 25° C. calculated from osmotic coefficients (Robinson and Stokes, 1955)

Molality	0·1	0·2	0·3	0·4	0·5
O.P.	4·57	9·06	13·55	18·04	22·56
Molality	0·6	0·7	0·8	0·9	1·0
O.P.	27·14	31·75	36·41	41·10	45·84

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An Endogenous Rhythm in the Rate of CO_2 Output of *Bryophyllum*

II. THE EFFECTS OF LIGHT AND DARKNESS ON THE PHASE AND PERIOD OF THE RHYTHM

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SUMMARY

The effects of light, darkness, and changes in light intensity on the phase and period of the endogenous rhythm in the rate of CO_2 output of excised leaves of *Bryophyllum fedtschenkoi* have been examined.

The duration, intensity, and wavelength of a short light treatment, and the point in the cycle at which it is administered, determine the degree of phase shift induced in a rhythm persisting in darkness. When light treatments of 3 and 6 hours' duration, at an intensity of 3,000 lux, are applied between the peaks the phase is completely reset, the first post-treatment peak occurring 18–19 hours after the end of the treatment. The degree of phase shift is therefore determined not by the duration of the treatment but by the time at which the treatment terminates. One hour's illumination has little or no effect. The phase is unaffected when light treatments of up to 5 hours' duration at an intensity of 3,000 lux are applied at the crest of a peak. Over the range 8–3,000 lux the intensity of light during a 6-hour treatment applied between the peaks does not affect the efficiency with which that treatment completely resets the phase. At an intensity of 2 lux, however, the phase delay is equal to the duration of the treatment.

A 6-hour red-light treatment (850 ergs/cm.^{2}/sec.) applied between the peaks completely resets the phase whereas blue light (10,860 ergs/cm.^{2}/sec.) has no effect on the phase but induces a slight protraction of the period. Moreover, continuous red light inhibits the rhythm, which recommences in blue light.

A rhythm is induced in illuminated leaves when the light intensity is either gradually or suddenly reduced by at least 80 per cent. Whether a given intensity of illumination inhibits or permits the persistence of a rhythm depends upon the light intensity by which it is immediately preceded.

A rhythm will persist in illuminated leaves for approximately as long as in leaves in darkness and the phase shows no correlation with time of day. The period is unaffected by the intensity of white light (from 0–500 lux) to which the leaves are subjected. The duration of a short dark treatment, and the point in the cycle at which it is applied, determine the degree of phase shift induced in a rhythm in illuminated leaves. The phase is reset when 3-, 6-, and 9-hour dark treatments are applied at the crest of a peak, the amount of phase shift increasing to a maximum with 9 hours' darkness. The phase shift is not equal to the duration of the treatment. The phase is unaffected when 3- and 6-hour dark treatments are applied between the peaks.

The variation in the sensitivity of the phase of a rhythm persisting in darkness to short light treatments is in the opposite sense to that of a rhythm persisting in

light to short dark treatments. The phase of a rhythm in illuminated leaves is completely reset when the leaves are transferred to continuous darkness commencing either at the crest of, or between, the peaks.

The results are discussed and compared with those of other authors.

INTRODUCTION

THE occurrence of a persistent endogenous rhythm in the rate of CO_2 output of excised leaves of *Bryophyllum fedtschenkoi* maintained in darkness, at a constant temperature and in an initially CO_2-free atmosphere, has been described by Wilkins (1958, 1959). It was found that the rhythm was inhibited by exposing leaves to a light intensity of 3,000 lux but recommenced when the light was extinguished. The time which elapsed between the restoration of darkness and the first peak (the transient) decreased as the duration of the light treatment increased. A more detailed investigation of the light inhibition of the rhythm seemed desirable. This paper describes the effect of short light treatments on the phase and period of a rhythm persisting in darkness and, following the observation that a rhythm could be induced in illuminated leaves, the effect of short dark treatments on the phase of a rhythm persisting in light.

The inhibition of endogenous rhythms by light has been recorded by Brandt (1953) in *Neurospora crassa*, by Uebelmesser (1954) and Schmidle (1951) in *Pilobolus*, and by Ingold and Cox (1955) in *Daldinia concentricia*. Bünsow (1953) found that a rhythm of petal movement in *Kalanchoë blossfeldiana* was strongly inhibited by light but that this inhibition could be reversed by exposing the flowers to a few hours' darkness. Ball and Dyke (1954) have shown that red light inhibited the rhythm in the growth-rate of *Avena* coleoptiles and Hastings and Sweeney (1957) found that while the rhythm of chemiluminescence in *Gonyaulax polyedra* was inhibited by light at an intensity of 800–1,600 f.c., transferring the culture to a lower light intensity (100–200 f.c.) or darkness reinitiated the rhythm. Bühnemann (1955) has shown that alternations of light of any wavelength and darkness induced persistent rhythms of sporulation and mitosis in *Oedogonium cardiacum*. Alternations of light of different wavelengths also induced rhythms; that part of the alternation containing the greater proportion of short-wavelength light acted as the dark phase.

Rhythms have been recorded which persist in light following transfer of the tissue from either darkness or a lower light intensity. Nuernbergk (1955) observed a rhythm in the rate of CO_2 assimilation in several species of succulent plants in light following alternations of light and darkness. Engel and Friederichsen (1951, 1952) induced a rhythm in the guttation rate of etiolated *Avena sativa* seedlings by transferring them from darkness to either continuous or intermittent light of any wavelength. Seedlings maintained in white light or darkness throughout were aperiodic, but an increase in the intensity of illumination would induce a rhythm as effectively as a change from darkness to light. Leinweber (1956) found that exposing seedlings of *Phaseolus multi-*

florus to a light intensity of 4,000–10,000 lux for 200 minutes initiated a rhythm in primary leaf movement which would persist in the low intensity yellow-green light in which the seedlings had been grown.

Bünning and Lörcher (1957) and Lörcher (1958) have shown that when plants of *Phaseolus multiflorus* are transferred from a greenhouse to darkness, or to wavelengths of light between 340–610 mμ. or above 850 mμ., they exhibited a 26·5-hour rhythm in primary leaf movement. Near-red light (610–90 mμ.) increased the period to 28 hours whereas far-red light (690–850 mμ.) decreased it to 24·3 hours. The rhythm persisted for a week in near-red light but for only a few days in far-red. Plants grown in continuous darkness, continuous light of any wavelength, and those transferred from near-red light to darkness were aperiodic, but transfer of etiolated plants from continuous darkness to near-red light induced a rhythm. A change from darkness to other wavelengths did not induce a rhythm.

The phase of a rhythm is initially set by a change from light to darkness or vice versa or by a change in the intensity of illumination. The phase of an established rhythm persisting under constant conditions may be reset by interrupting the constant conditions for a few hours. Kleinhoonte (1932) observed that the phase of the rhythm of leaf movement in *Canavalia ensiformis* could be displaced by 12 hours by applying a short light treatment when the leaves were in the 'sleep' position. Bünning and Lörcher (1957) found that the induction of a rhythm of leaf movement in etiolated plants of *Phaseolus multiflorus* by 12 hours' exposure to near-red light was suppressed by 12 hours' far-red light only when the far-red treatment immediately followed the near-red treatment. Application simultaneously or after a 12-hour interval gave no suppression, showing that far-red only prevents the induction of a rhythm when applied to the plants during that part of the cycle which normally occurs at night. In papers published as the present work was being completed Hastings and Sweeney (1957, 1958) recorded that the degree of phase shift induced by a short light treatment in the rhythm in *Gonyaulax polyedra* depends upon the duration, intensity, and wavelength of the treatment, and the point in the rhythm at which it is administered.

In several cases the persistence of a rhythm is unaffected by the nature of the constant conditions in which the tissue is maintained. Rhythms in the rate of O_2 metabolism in *Hydrodictyon reticulatum* (Schön, 1955) and in the change in plastid shape in *Selaginella serpens* (Busch, 1953) persist for a similar time in either continuous light or darkness.

MATERIAL AND METHODS

Plants of *Bryophyllum fedtschenkoi* were grown in a heated greenhouse under short-day illumination from 8 a.m. until 4 p.m. The method of growing the plants and the experimental technique have been described by Wilkins (1959) and more fully by Wilkins (1958). The excised leaves were placed in a specially constructed chamber which was immersed 18 cm. below the surface of a large water bath maintained at a temperature of 26° C. In all experiments

the leaves were kept in an initially CO_2-free air stream and their rate of CO_2 output was measured with an infra-red gas analyser. Illumination was provided by tungsten lamps, fine adjustment of intensity being facilitated by a large resistance in series with the lamps. Light of various wavelengths was obtained by placing a double layer of 'Cinemoid' filter between the light source and the material. The transmission curves for single layers of the filters are shown in Fig. 1. The intensity of white light was measured with an Everett-Edgecombe autophotometer, while the intensity of radiation of coloured light was measured with a calibrated thermopile in conjunction with a Vernier potentiometer.

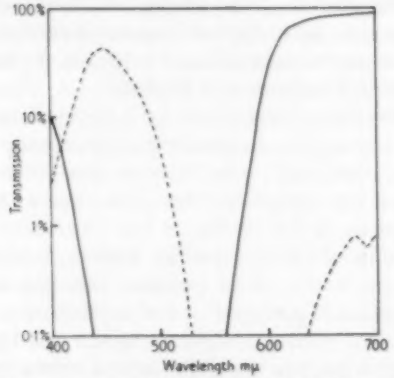

FIG. 1. Shows the transmission curves of the 'Cinemoid' filters. Continuous line = No. 6 (red). Broken line = No. 20 (blue).

RESULTS

A control rhythm in the rate of CO_2 output of excised leaves of *Bryophyllum fedtschenkoi*, shown by the broken line in Figs. 2A to 2D and 3A to 4B, was induced in the following manner. Leaves were detached from the plant at 3 p.m. and placed in an initially CO_2-free air stream and a light intensity of 3,000 lux. At 4 p.m. the light was permanently extinguished and a rhythm commenced in which the first peak occurred at 11 a.m. the following morning, 19 hours after the commencement of darkness. Subsequent peaks occurred at approximately 22-hour intervals.

I. *The Effect of Short Light Treatments on a Rhythm Persisting in Darkness*

1. *The Duration and Position of the Treatment*

(*a*) *The effect of a treatment between the peaks.* The effect of exposing leaves to short light treatments at an intensity of 3,000 lux and of various durations between the first and second peaks of a rhythm persisting in darkness is shown in Figs. 2A–E. Illuminating leaves for 6 hours (from 8 p.m. until 2 a.m., Fig. 2A) or 3 hours (from 11 p.m. until 2 a.m., Fig. 2B) completely reset the phase of the rhythm; the first post-treatment peaks occurred at

7.30 p.m. and 10 p.m., 17·5 and 20 hours after the end of the treatments respectively. One hour's illumination from either 1 a.m. to 2 a.m. (Fig. 2C) or 9 p.m. to 10 p.m. (Fig. 2D) did not completely reset the phase but slightly reduced the periods between the first, second, and third peaks to 20–21 hours. Moreover, a further experiment revealed that the phase was not reset even

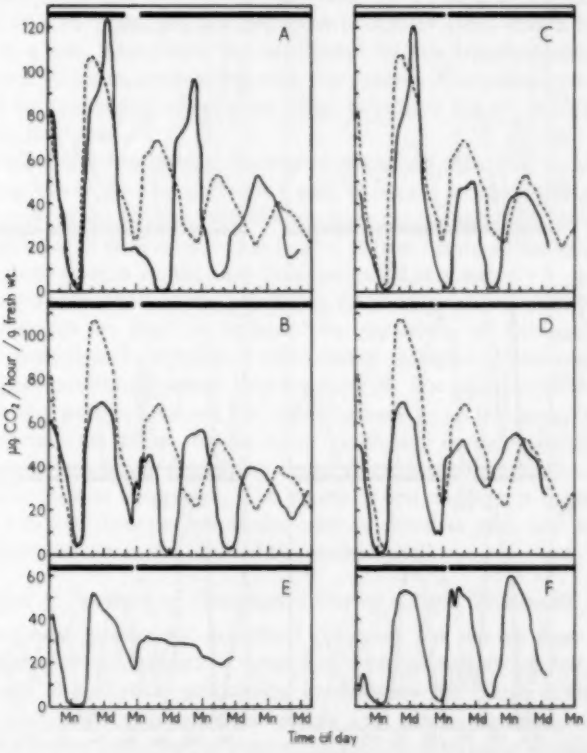

FIG. 2. Shows the effect of short light treatments on the phase of a rhythm persisting in darkness. The illumination programme is shown by the bar above each graph. The broken line indicates a control experiment in continuous darkness. Ordinates: μg. CO_2/hr./g. fresh weight; Abscissae: time of day, Mn. = midnight and Md. = midday. For explanation see text.

when the leaves were illuminated for one hour from 1 a.m. to 2 a.m. at an intensity of 20,000 lux (Fig. 2F) although the period was again slightly decreased. One hour's illumination at an intensity of 3,000 lux from 11 p.m. until midnight did, however, produce a slight shift in phase. The first post-treatment peak would have been expected at 6 p.m. if the phase had been completely reset and at 9 a.m. if the original phase had been maintained. The rather flattened peak in Fig. 2E occurred mid-way between these alternative positions,

indicating that the curve may be the resultant of two rhythms differing in phase by approximately 8 hours.

Exactly similar results were obtained when these experiments were repeated using 1 cm.^{2} pieces of mesophyll which had been stripped of their epidermis.

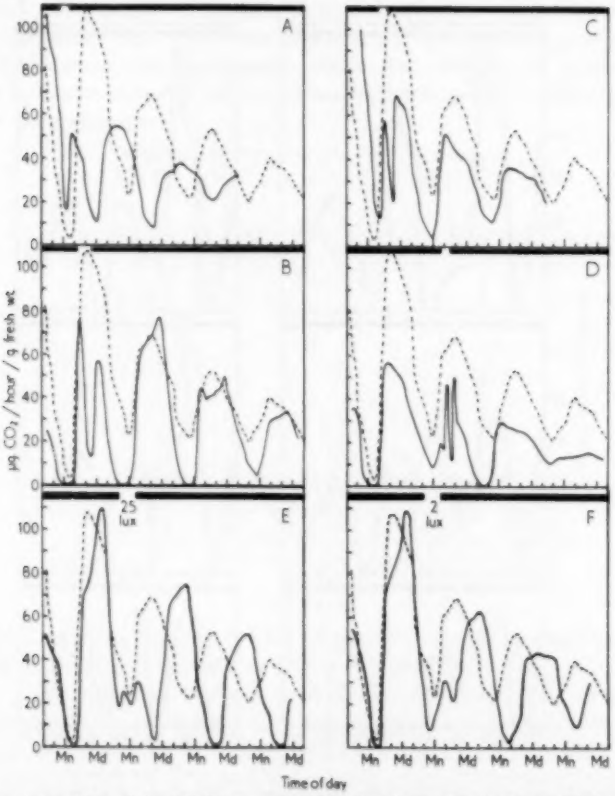

FIG. 3A–D. The effect of short light treatments on the phase of a rhythm persisting in darkness. Control experiment in continuous darkness shown by the broken line. For explanation see text.

FIG. 3E–F. The effect of intensity of illumination during a 6-hour light treatment applied between the peaks. Control experiment in continuous darkness shown by the broken line. For explanation see text.

(*b*) *The effect of a treatment in the trough before the first peak.* Fig. 3A shows that the phase of a rhythm was completely reset by illuminating leaves for 3 hours at an intensity of 3,000 lux from 11 p.m. until 2 a.m. in the trough before the first peak. The first post-treatment peak occurred at 8.30 p.m., 18·5 hours after the end of the treatment. This result is closely similar to that of exposing leaves to 3 hours' illumination between the first and second peaks.

(*c*) *The effect of a treatment at the crest of the first peak.* Leaves were subjected to light treatments of 5, 3, and 1 hours' duration at an intensity of 3,000 lux at the crest of the first peak of a rhythm persisting in darkness. Five hours' illumination at the crest of a peak was the maximum treatment which permitted a clear indication of whether or not the phase had been completely reset. If the phase had been completely reset by 5 hours' illumination from 5 a.m. to 10 a.m. (Fig. 3B) the first post-treatment peak would have been expected at 4 a.m. The phase was unaffected by this treatment; the peak in fact occurred at 9 a.m., coinciding with the control. The phase was similarly unaffected by treatments of 3 hours' (Fig. 3C) and 1 hour's duration at the crest of the first peak.

(*d*) *The effect of a treatment at the crest of the second peak.* Fig. 3D shows that illuminating leaves for 3 hours from 3 a.m. to 6 a.m. at an intensity of 3,000 lux at the crest of the second peak did not reset the phase of the rhythm. The first post-treatment peak occurred at 6 a.m., within 1 hour of the control; had the phase been reset it would have been expected at 1 a.m.

The results described in the preceding four sub-sections clearly indicate that when leaves are kept in darkness the sensitivity of the phase of the rhythm to short light treatments of a few hours' duration varies according to the point in the rhythm at which they are applied. The phase is most sensitive to light in the trough between the peaks, where 3- and 6-hour treatments completely reset the phase, but is either unaffected or very slightly shifted by 1 hour's illumination depending upon the exact point in the trough at which the treatment is applied. The phase of the rhythm is insensitive to 5, 3, and 1 hours' illumination at the crest of the first peak and to at least 3 hours' illumination at the crest of the second peak.

2. *The Effect of Intensity of Illumination During a Light Treatment*

An experiment previously described (Fig. 2A) has shown that if leaves are illuminated at an intensity of 3,000 lux for 6 hours between the first and second peaks of a rhythm proceeding in darkness the phase is completely reset; the first post-treatment peak occurs 17·5 hours after the end of the treatment. Further experiments were conducted to determine whether or not the intensity of illumination during a 6-hour light treatment, applied to the material between the peaks from 8 p.m. until 2 a.m., had any effect upon the efficiency with which that treatment completely reset the phase.

Light intensities of 500, 25 (Fig. 3E), and 8 lux completely reset the phase, the first post-treatment peaks occurring 19, 17, and 17·5 hours after the end of the treatments respectively. In Fig. 3F, where the material was illuminated at an intensity of 2 lux, the phase was not completely reset; the first post-treatment peak occurred only 13·5 hours after the end of the treatment. The phase was in fact delayed by 6 hours, a time equal to the duration of the treatment. In a further experiment a phase delay of 12 hours resulted from illuminating leaves for 12 hours at an intensity of 2 lux.

Over the intensity range 8–3,000 lux the intensity of illumination during

a 6-hour light treatment has no effect on the efficiency of that treatment in completely resetting the phase. The amount of phase shift induced is therefore determined by the time at which the treatment terminates. At an intensity of 2 lux, however, the phase delay equals the duration of the light treatment.

3. *The Effect of Wavelength of Illumination During a Light Treatment*

Leaves were illuminated with light of various wavelengths for 6 hours from 8 p.m. until 2 a.m. in the trough between the first and second peaks of a rhythm persisting in darkness. The spectrum was divided, by means of filters,

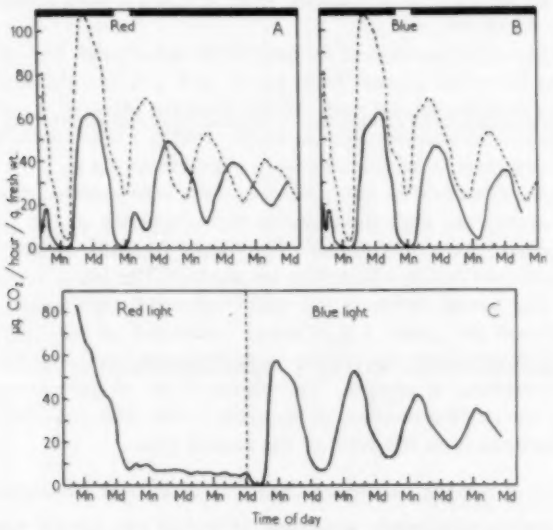

FIG. 4. The effect of light of different wavelengths on the rhythm. Control in continuous darkness shown by the broken line. For explanation see text.

into red (above 565 mμ.) and blue (below 525 mμ.) portions and the effect of each portion was determined at two intensities.

The effect of illuminating the material with a low-intensity red light (850 ergs/cm.²/sec.) is shown in Fig. 4A. The phase of the rhythm was completely reset; the first post-treatment peak occurred at 7 p.m., 17 hours after the end of the treatment. A closely similar result was obtained by illuminating leaves with red light for 6 hours at an intensity of 3,620 ergs/cm.²/sec. Illuminating the material with blue light (10,860 ergs/cm.²/sec.) did not reset the phase (Fig. 4B). The initial peak of the rhythm occurred at 12.30 p.m., 1·5 hours later than the control. The second peak occurred 23 hours after the first and the third 24 hours after the second. Similar application of blue light at an intensity of 1,960 ergs/cm.²/sec. produced an identical result.

In a further experiment (Fig. 4C) leaves which had been subjected to 68 hours' red light (16,430 ergs/cm.²/sec.) were suddenly transferred to con-

tinuous blue light (10,860 ergs/cm.²/sec.) at midday. The rhythm was inhibited by the red light but recommenced in blue light; the first post-treatment peak occurred at 2 a.m., 14 hours after the transfer. Subsequent peaks occurred at approximately 25·5-, 24- and 23-hour intervals.

The results in this section show that wavelengths of light below 525 mμ. do not inhibit the rhythm but may, in some cases, cause a slight protraction of the period, whereas wavelengths of light above 565 mμ. completely inhibit the rhythm. Transferring leaves from red light to either darkness or blue light initiated a rhythm in which the first peak occurred a specific time after the transfer depending upon the duration of the red-light treatment. This was in close agreement with the relation between the duration of a continuous white-light treatment and the transient, shown by Wilkins (1959).

An experiment in which leaves were illuminated for 6 hours between the peaks by light of wavelengths between 475 mμ. and 575 mμ. (green) and at an intensity of 6,880 ergs/cm.²/sec. revealed that the phase was not completely reset by this treatment. A phase delay of 4·5 hours was recorded, showing an effect intermediate between those of red and blue light.

II. *The Effect of Changes in the Intensity of Illumination on the Rhythm*

(*a*) *Sudden changes.* A series of experiments was carried out to determine the effect of a single instantaneous change in the intensity of illumination upon the phase of the rhythm.

Fig. 5A shows that when leaves were placed in darkness at 4 p.m. a rhythm commenced in which the first peak occurred at 11 a.m., 19 hours after the commencement of darkness. At 8 p.m., after the rhythm had proceeded in darkness for 52 hours, the material was illuminated at an intensity of 500 lux. The rhythm continued for 4 to 5 hours but subsequently the rate of CO_2 output decreased to a low constant value. After 40 hours' illumination the light was extinguished at noon and the rhythm recommenced; the first peak occurred 14 hours after the end of the light treatment. Clearly, light at this intensity, when preceded by darkness, strongly inhibits the rhythm.

Fig. 5B shows the effect of illuminating leaves at an intensity of 2,500 lux for 25 hours commencing at 4 p.m. A low, approximately constant rate of CO_2 output was recorded with no indication of a rhythm. At 5 p.m. the light intensity was suddenly reduced to 500 lux and, although no immediate rise in the rate of CO_2 output was recorded, a rhythm commenced in which the first peak occurred at 7.30 a.m., 14·5 hours after the intensity reduction. The rhythm persisted with a 22-hour period for 51 hours then, at 8 p.m., the light intensity was further reduced to 25 lux. Now, assuming that no change of phase had occurred the expected positions of subsequent peaks could be calculated from the phase of the rhythm in 500 lux. These would have occurred at 2 a.m. and midnight (22 hours later). If the phase had been completely reset by the change from 500 lux to 25 lux the peaks would have occurred at approximately 10 a.m., 14 hours after the change, and 8 a.m. (22 hours later) (see Wilkins, 1959). Examination of the graph following the

second intensity change reveals that the phase coincides with neither of these alternatives but that the peaks occurred at 6 a.m. and 3.30 a.m. (21·5 hours later), mid-way between the theoretical alternatives. In view of the high over-all rate of CO_2 output and the decreased amplitude, the rhythm exhibited by the leaves after the change from 500 to 25 lux must be the resultant of two rhythms proceeding in the phases outlined above. This apparent demonstration of two rhythms proceeding slightly out of phase in pieces of tissue (and

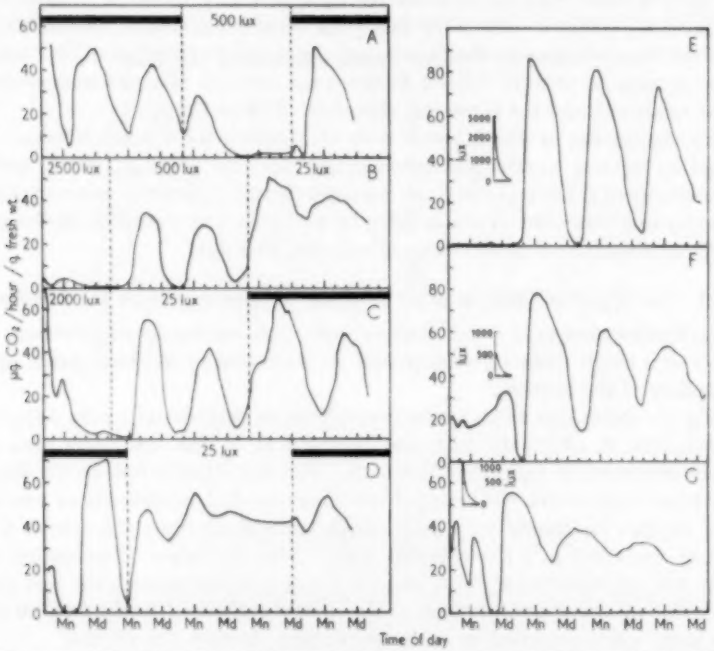

FIG. 5A–D. Shows the effect of changes in the intensity of illumination on the rhythm.

FIG. 5E–G. Shows the effect of gradually reducing various initial intensities of illumination to darkness at the rate and time of day shown by the graphs inset on the main figures. For explanation see text.

possibly in the same piece of tissue) that have received identical treatment poses the question of whether some of the cellular 'clocks' have become desynchronized or whether further 'clocks' have been created *de novo*.

The results of two further experiments, carried out using different light intensities, fully confirm these results. In Fig. 5C leaves were illuminated at an intensity of 2,000 lux for 25 hours commencing at 4 p.m. The rate of CO_2 output gradually decreased showing no rhythmic fluctuations. At 5 p.m. the light intensity was reduced to 25 lux, when a rhythm commenced in which the first peak occurred at 7.30 a.m., 14·5 hours after the intensity change. The rhythm persisted with a 22-hour period; the second peak occurred at 5.30 a.m.

and a third peak would clearly have occurred at approximately 3.30 a.m. After 51 hours' illumination at 25 lux the light was extinguished. The phase was completely reset by this change; the first peak occurred at 9 a.m., 13 hours after the end of the light treatment, and a second 24 hours later. In Fig. 5D leaves were placed in darkness for 31 hours commencing at 4 p.m. and subsequently illuminated for 61 hours at an intensity of 25 lux. The normal rhythm exhibited in darkness was inhibited by the light treatment though some random fluctuations occurred during the first 30 hours' illumination. After 61 hours' illumination the light was extinguished and a rhythm commenced in which the first peak occurred at 1 a.m., 13 hours after the commencement of darkness.

The effect of a given intensity of light on the rhythm depends upon the intensity of illumination by which it is immediately preceded. Light intensities of 500 and 25 lux completely inhibit the rhythm if preceded by darkness but if these intensities are preceded by higher intensities of 2,500 and 2,000 lux respectively a rhythm is initiated by the transfer and persists with a phase and period closely similar to those of a rhythm proceeding in darkness. Over the range 0–500 lux the period of a rhythm (at 26° C.) in illuminated leaves is not a function of the light intensity.

A rhythm will persist in leaves maintained in a low-intensity light for approximately as long as in leaves maintained in darkness and the phase shows no correlation with time of day. Leaves were illuminated for 25 hours at an intensity of 2,500 lux which was suddenly reduced to 500 lux at 5 p.m. A rhythm commenced in which the first peak occurred at 9 a.m. When leaves were illuminated for 13 hours at an intensity of 3,000 lux which was reduced to 100 lux at 5 a.m. (Fig. 7A and 7E, broken lines) a rhythm commenced in which the first peak occurred at 9 p.m., completely out of phase with the rhythm in the previous experiment. In both experiments the period was approximately 22 hours.

A significant point to emerge from these results is the ability of a single stimulus to initiate a 22-hour rhythm in the leaves. Throughout a light treatment the material retains the potentiality for an accurately functioning 22·5-hour rhythm.

(*b*) *Gradual changes.* Further experiments were carried out in which various initial intensities of illumination were gradually reduced to darkness over a period of 6 hours. The rate of intensity reduction is shown by the small curves inset on Figs. 5E, F, and G.

Fig. 5E shows the result of illuminating leaves at an intensity of 3,000 lux from 4 p.m. until 9 a.m. the following morning. From 9 a.m. until 3 p.m. the intensity was gradually reduced to darkness which continued for the remainder of the experiment. No immediate change in the rate of CO_2 output was recorded, but at 7 p.m. a sudden increase occurred, resulting in a clearly defined 23-hour rhythm in which the first peak occurred at 3.30 a.m.

In order to determine the time at which the rhythm commenced it is necessary to consider the length of time for which the leaves had been

illuminated. The relation between the duration of a continuous light treatment and the time which elapses between the commencement of darkness and the first peak (the transient), described by Wilkins (1959), shows that following approximately 20 hours' continuous illumination since the experiment was set up the first peak would be expected 15·7 hours after the rhythm commenced. A point preceding the first peak of the rhythm in Fig. 5E by 15·7 hours corresponds to a time at which the light intensity had reached a value of approximately 600 lux.

Similar experiments were carried out using initial intensities of 2,000, 1,000, 500 (Fig. 5F), and 100 lux, which were gradually reduced to darkness from 9 a.m. until 3 p.m. In all cases rhythms were initiated and points preceding the first peaks by 15·7 hours corresponded to times at which the light intensities had reached values of approximately 400, 200, 100, and 20 lux respectively. In a further experiment (Fig. 5G) in which the initial light intensity of 1,000 lux was reduced to darkness from 9 p.m. until 3 a.m. a rhythm commenced in which the first peak occurred at 5 p.m. Following approximately 8 hours' illumination since the experiment was set up the first peak would be expected 17 hours after the commencement of the rhythm (see Wilkins, 1959). A point preceding the first peak by 17 hours corresponds to a time at which the intensity had reached a value of 200 lux. Clearly, the phase of the rhythm induced in this way is entirely independent of time of day.

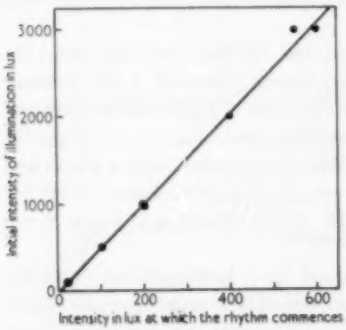

FIG. 6. Shows the linear relation between the initial intensity of illumination to which the leaves were subjected and the intensity at which the rhythm commenced. For explanation see text.

These results show that when the light intensity is gradually reduced there is an approximately linear relation between the initial light intensity to which the leaves are subjected and the intensity at which the rhythm commences (Fig. 6). An intensity reduction of 80 per cent. is necessary to initiate a rhythm rather than reduction below a critical value. Gradual reduction of the light intensity from 2,000 lux to 500 lux did not induce a rhythm in leaves but subsequent transfer to darkness did initiate a rhythm, thus confirming the above results.

III. *The Effect of Short Dark Treatments on the Phase of a Rhythm Proceeding in Light*

Leaves which had been transferred from a light intensity of 3,000 lux to one of 100 lux exhibited a rhythm which persisted for at least 104 hours, showing 5 clearly defined peaks (Figs. 7A and E, broken lines). The intensity was reduced at 5 a.m. and the first peak occurred at 9 p.m. Experiments were

conducted to determine the effect of interrupting the continuous illumination with short periods of darkness at various points in the rhythm.

(*a*) ***Dark treatments at the crest of a peak.*** Fig. 7A shows the effect of subjecting leaves to continuous darkness from 8 p.m. at the crest of the first peak of a rhythm proceeding in 100 lux. The phase was completely reset; the first post-treatment peak occurred at 11.30 a.m., 15·5 hours after the onset of

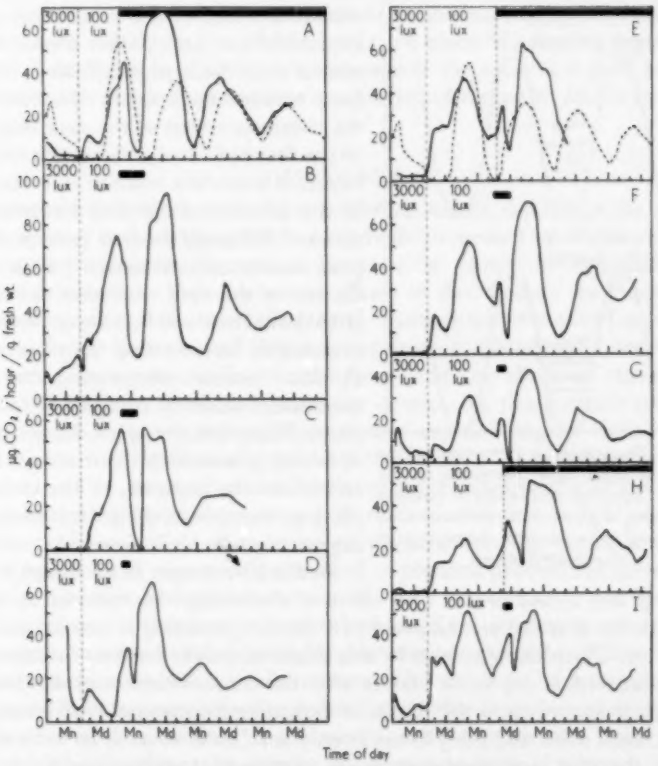

FIG. 7. The effect of darkness on the phase of a rhythm persisting in illuminated leaves. Illumination programme shown by the bar above each graph. Broken line shows the control rhythm in leaves illuminated at an intensity of 100 lux throughout. For explanation see text.

darkness. The phase was also completely reset by subjecting the leaves to 9 hours' darkness from 8 p.m. until 5 a.m. (Fig. 7B); the first post-treatment peak occurred 15·5 hours after the commencement of, or 6·5 hours after the end of, the dark treatment.

Darkening the leaves for 6 hours, from 8 p.m. until 2 a.m. (Fig. 7C), or for 3 hours, from 8 p.m. until 11 p.m. (Fig. 7D), induced a phase shift; the first

post-treatment peaks occurred 13 and 10·5 hours after the commencement of, or 7 and 7·5 hours after the end of, the dark treatments respectively.

Clearly the phase is reset by continuous 9, 6, and 3 hours' darkness commencing at 8 p.m. at the crest of a peak and in the case of 9, 6, and 3 hours' darkness the first post-treatment peak occurs approximately 7 hours after the end of the treatment. In the cases of continuous and 9 hours' darkness the first peaks occurred 15·5 hours after the commencement of the treatments, a time expected from the relation shown in the author's previous paper. With 6- and 3-hour treatments, however, the time from the commencement of the dark treatment to the first peak does not coincide with that expected from this relation but decreases as the duration of the dark treatment decreases. Although the first post-treatment peak occurs approximately 7 hours after the end of the dark treatment the change from darkness to 100 lux cannot be entirely responsible for resetting the phase of the rhythm because transferring leaves to continuous darkness completely reset the phase. It appears, therefore, that in the case of 6- and 3-hour dark treatments the end, and hence the duration, of the treatment plays an important role in determining the degree of phase shift obtained.

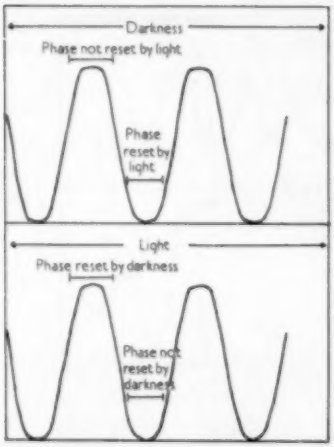

FIG. 8. Diagrammatic comparison of the effects of short light treatments on the phase of a rhythm proceeding in darkness with those of short dark treatments on the phase of a rhythm persisting in illuminated leaves.

(*b*) *Dark treatments in the trough between the first and second peaks.* The effect of darkening the material at 7 a.m. between the first and second peaks of a rhythm persisting in 100 lux is shown in Fig. 7E. The phase was reset by this single stimulus; the first post-treatment peak occurred at 9 p.m., 14 hours after the commencement of darkness.

Figs. 7F and 7G show the results of darkening the material for 6 hours from 6 a.m. until noon and for 3 hours from 7 a.m. until 10 a.m. In both experiments the first post-treatment peaks occurred at approximately 6 p.m., 22 hours after the previous peaks and coinciding with the control. Clearly the phase was not reset by these treatments, but in view of the small time interval (3 hours) between the expected position of the first post-treatment peak when the maximum degree of phase shift is elicited and its position if the original phase is maintained, two further experiments were conducted in which the timing of the dark treatment was such that a longer interval would elapse between the two alternative positions of the first post-treatment peak.

In Fig. 7H continuous darkness commenced at 9 a.m. and the phase was completely reset; the first post-treatment peak occurred at 11.30 p.m., 14·5 hours after the commencement of darkness. When the material was subjected

to 3 hours' darkness from 9 a.m. until noon (Fig. 7*i*) the first post-treatment peak occurred at 7 p.m., 22·5 hours after the previous peak. Clearly the phase is unaffected by 6- and 3-hour dark treatments applied between the peaks.

These results clearly indicate that the sensitivity of the phase of a rhythm proceeding in light to short dark treatments varies according to the point in the rhythm at which they are applied. The most significant result to emerge from this investigation is that whereas in a rhythm proceeding in darkness the phase is most sensitive to light treatments between the peaks and is insensitive to light treatments at the crests of the peaks, the phase of a rhythm proceeding in light is sensitive to short dark treatments at the crest of a peak but not between the peaks. A diagrammatic comparison of these two effects is shown in Fig. 8.

DISCUSSION

Further investigation of the light inhibition of the rhythm in the rate of CO_2 output of excised leaves of *B. fedtschenkoi*, reported by Wilkins (1959), has revealed a complex interaction between the effects of light, darkness and changes in light intensity on the phase of the rhythm. As Pittendrigh and Bruce (1957), Ball and Dyke (1957), Hastings and Sweeney (1958), and Bünning (1958) have pointed out, it is necessary to distinguish between the basic time-keeping mechanism in the tissue and the observed rhythms in metabolic and other processes under its control. In the author's previous paper (1959) a simple model oscillating system, representing the basic 'clock', was introduced to facilitate discussion of the results; use will again be made of this model to summarize and condense the characteristics of the endogenous time-keeping mechanism and its reactions to various light and dark treatments. Nevertheless, it must be emphasized that the model is not intended to represent any specific chemical or physical process but merely the unknown 'clock' mechanism in the tissue.

The variation in the sensitivity of the phase of the rhythm to 3-hour light treatments at an intensity of 3,000 lux according to the point in the rhythm at which they are applied is an observation of fundamental importance. It has previously been suggested by the author (1959) that the effect of continuous illumination on the postulated model system oscillating between limits *A* and *B* is to inhibit oscillation by driving the system to limit *A* where it is held until the light is extinguished. Now in the freely oscillating system there would be times when the system was at or near *A*, when application of a short light treatment would have no effect upon the subsequent phase, and times when it was near limit *B* when a short light treatment would drive the system to limit *A* and hold it there for the duration of the treatment. The oscillations would recommence on the restoration of darkness by the system moving towards *B* and the first post-treatment peak would occur a specific time after the end of the treatment. Moreover, this situation accounts for the fact that the degree of phase shift is neither equal nor proportional to the duration of a light treatment applied between the peaks (over the intensity range 8–3,000

lux) but is determined by the time at which the treatment terminates. Hastings and Sweeney (1957, 1958) induced a maximum phase shift in the rhythm of luminescence in *Gonyaulax* by applying a 3-hour light treatment (1,400 f.c.) at the crest of a peak; little or no phase shift was induced by applying similar treatments between the peaks. Bünning and Lörcher (1957) have shown that far-red light suppresses the induction of a rhythm in leaf movement in *Phaseolus multiflorus* by near-red light only when it is applied during the 'dark' part of the cycle.

The duration of a light treatment at an intensity of 3,000 lux applied between the peaks is an important factor in determining whether or not the phase is reset. A critical duration of between 1 and 3 hours must be exceeded for the phase to be completely reset so that the first post-treatment peak occurs 18–19 hours after the end of the treatment. One hour's illumination, even at an intensity as high as 20,000 lux, had no effect upon the phase when applied at several points between the peaks although a slight decrease in the period was noted. At one point, however, 1 hour's illumination at an intensity of 3,000 lux resulted in a slight phase shift. These results contrast with those of Kleinhoonte (1932), who found that the phase of the leaf movement rhythm in *Canavalia ensiformis* could be displaced by 12 hours with 1 minute's illumination when the leaves were in the 'sleep' position providing the intensity was sufficiently high. In the *Gonyaulax* rhythm the degree of phase shift was proportional to the duration of a light treatment applied 6 hours after the cells were placed in darkness, reaching a maximum of 11·5 hours with 2·5 hours' illumination (Hastings and Sweeney, 1957, 1958).

The light intensity, over the range 8–3,000 lux, during a 6-hour treatment applied between the peaks had no effect upon the efficiency with which that treatment completely reset the phase of the rhythm; the first post-treatment peak always occurred approximately 18 hours after the recommencement of darkness. At an intensity of 2 lux, however, the phase delay was equal to the duration of the treatment. The light treatments always commenced approximately 4 hours before the rate of CO_2 output reached a minimum value, that is, in terms of the freely oscillating model 'clock', when the system was approaching limit *B*. These results clearly support the hypothesis that the oscillating system is driven to and held at limit *A* by light in the intensity range 8–3,000 lux. At an intensity of 2 lux it appears that the system is not driven to limit *A* but is merely 'frozen' in the position it had reached when the light stimulus was applied. On the restoration of darkness oscillations recommenced by the system moving towards limit *B*, thus resulting in a phase delay equal to the duration of the treatment. Hastings and Sweeney (1957, 1958) found that the degree of phase shift induced in the rhythm in *Gonyaulax* increased linearly with increasing intensity during a short 2·5-hour light treatment up to a saturation point at 800 f.c. Obviously in *Bryophyllum* such a saturation point must lie between 2 and 8 lux.

Wavelengths of light above 565 mμ. inhibit the rhythm when applied to the leaves continuously and completely reset the phase when applied in 6-hour

treatments between the peaks of a rhythm proceeding in darkness. On the other hand, wavelengths below 525 mμ. presented under similar circumstances neither inhibit the rhythm nor reset the phase although a slight protraction of the period was noted. The phase is reset by 6 hours' red light at an intensity as low as 850 ergs/cm.²/sec., but is unaffected by 6 hours' blue light at an intensity of 10,860 ergs/cm.²/sec. In contrast, Bünning and Lörcher (1957) and Lörcher (1958) found red light to be most effective for modifying the period of the rhythm in *Phaseolus*; blue light had no effect. However, Bühnemann (1955) has shown that whereas blue light acted as the 'light' phase in alternation with darkness it acted as the 'dark' phase in alternation with longer wavelengths of light for inducing a rhythm of sporulation in *Oedogonium*. Light of wavelength 650 mμ. induced a phase shift in the *Gonyaulax* rhythm but a shift was also induced by light of wavelength 475 mμ. (Hastings and Sweeney, 1957). While Ball and Dyke (1954) have shown that the rhythm in the growth rate of *Avena* coleoptiles was inhibited by red light, Engel and Friederichsen (1951, 1952) found, on the other hand, that the guttation rhythm in *Avena* coleoptiles was induced by transferring the seedlings from darkness to light of any wavelength.

The study of the effect on the rhythm of a single change in the light intensity revealed that a given intensity may permit or inhibit the persistence of a rhythm depending upon the intensity by which it is immediately preceded. In order to initiate a rhythm in illuminated leaves the light intensity may be either suddenly or gradually reduced; the rhythm commences, and the phase is set, when the intensity has been reduced by at least 80 per cent. Rhythms have been induced in the luminescence of *Gonyaulax* (Hastings and Sweeney, 1957), the guttation rate of *Avena* seedlings (Engel and Friederichsen, 1952) and the leaf movement of *Phaseolus multiflorus* (Leinweber, 1956) by changes in light intensity, and Bünning (1935) was able to induce a rhythm of eclosion in *Drosophila* in weak light by exposing the pupae to light of a higher intensity for a few hours.

The period of the *Bryophyllum* rhythm (at 26° C.) is not a function of the intensity of white light to which the leaves are subjected. The period is approximately 22 hours irrespective of whether the leaves are maintained in continuous darkness or light intensities of 25, 100, or 500 lux. This contrasts with the slight protraction of the period observed when the leaves were subjected to light of wavelength below 525 mμ. Hastings and Sweeney (1957, 1958) have shown that the light intensity to which cultures of *Gonyaulax* were exposed modified the period of the rhythm of luminescence at 21° C. by only 1–2 hours from 24·5 hours in darkness to 22·0 hours at 680 f.c.

The induction of phase shifts in the *Bryophyllum* and *Gonyaulax* rhythms is closely similar in that a new stable phase is reached immediately. There is no indication, as in the rhythm in *Drosophila* (Pittendrigh and Bruce, 1957), of a new stable phase being reached only after several days.

The final series of experiments show that the phase of a rhythm proceeding in light varies in its sensitivity to short dark treatments according to the point

in the rhythm at which they are administered. The phase was reset by treatments applied at the crest of a peak but not between the peaks. This variation was in the opposite sense to that of a rhythm proceeding in darkness and subjected to short light treatments. The degree of phase shift induced by a short dark treatment applied at the crest of a peak is determined by the duration of the treatment, increasing to a maximum with 9 hours' darkness. These results would suggest that in the postulated model system oscillating in light the onset of darkness at the crest of a peak when the system is near limit *A* in some way induces the system to move towards limit *B*. Short 3- or 6-hour dark treatments applied when the freely oscillating system is near limit *B*, that is between the peaks in the rate of CO_2 output, should therefore produce no phase shift; that this is so is confirmed by experiment. The induction of maximum phase shift by continuous and 9 hours' darkness commencing at the crest of a peak indicates that in these cases the system must have travelled from *A* and actually reached limit *B*. For dark treatments of 3 and 6 hours' duration, however, the degree of phase shift became less as the duration of the treatment decreased, suggesting that the length of the treatment permitted the model system to move only part of the way to limit *B*.

Two fundamental differences between the effects of light and darkness on such a model 'clock' now become evident. Firstly, when leaves having a rhythm proceeding in light are transferred to continuous darkness the phase is completely reset but the rhythm is never inhibited. Clearly, darkness can only induce the system to move towards *B* but has no power to hold the system at *B* in a manner comparable to that in which light inhibits a rhythm by holding the system at *A*. Secondly, movement of the system from *B* to *A* by light is complete in under 3 hours whereas movement of the system from *A* to *B* in darkness takes between 6 and 9 hours (determined by the minimum treatment required to completely reset the phase, i.e. to induce maximum phase shift for treatments terminating or commencing at a given time). It seems probable that light energy activates the *B* to *A* process via the leaf pigments which absorb wavelengths of light above 565 mμ. whereas movement from *A* to *B* is a relatively passive 'dark' process.

A particularly difficult phenomenon to explain is that of a phase shift induced when leaves having a rhythm persisting in light are exposed to continuous darkness commencing between the peaks where a 6-hour dark treatment has no effect upon the subsequent phase. Elaboration of the simple model 'clock' which has been postulated is not justified until a more detailed study of this phenomenon has been made. The change induced by a single stimulus in the phase of a biological rhythm provides one of the most important outstanding problems in this field. Bünning (1958) has recently suggested that a change in the axis of oscillation of the 'clock', considered by him to be a type of relaxation oscillator, could account for a phase shift induced in the rhythms in several organisms by a single temperature change. If, in *Bryophyllum*, reduction in the light intensity by 80 per cent. results in the oscillations of the 'clock' being about a slightly different axis to those of the

'clock' in darkness then the process whereby the axis subsequently moves from the 'light' level to the 'dark' level when the light is extinguished requires at least 6 hours' darkness. Furthermore, the shift in the axis of oscillation would also occur when continuous darkness commenced at the crest of a peak and the resulting phase shift would be due to two effects: movement of the system from *A* to *B* as previously outlined and the gradual movement of the axis of oscillation. Whatever the actual mechanism some difference clearly exists between the characteristics of the oscillations of the 'clock' in light and in darkness.

Some change in the sensitivity of the rhythm to light was expected following the application of short dark treatments to leaves having a rhythm persisting in light. A rhythm proceeding in darkness is strongly inhibited by light but a rhythm proceeding in a light intensity of 100 lux was not completely inhibited by re-exposure to that intensity following 3-, 6-, and 9-hour dark treatments. The amplitude was, however, low and the rhythm became irregular and indistinct after the second post-treatment peak. Clearly, the change in the tissue from a state which permits the persistence of a rhythm in light to one that causes the same intensity to inhibit the rhythm requires approximately 6–9 hours' darkness. If this change involves some readjustment in the axis of oscillation of the endogenous 'clock' it should be accompanied by a phase shift. That the induction of a change in the sensitivity of the tissue to light and of a phase shift by darkness commencing between the peaks require at least 6 hours' darkness may indicate that these phenomena are controlled by a common mechanism.

Finally, several curves presented in this paper have the appearance of being the resultant of two rhythms of different phase. The evidence for this has been stated in a previous section, and while no specific investigation has been made of this problem the occurrence of such curves supports the suggestions of Pittendrigh (1954), Bünning (1956), and Ball and Dyke (1957) that the 'clocks' in the cells of an organism are normally synchronized by changes in external conditions.

The reactions of the rhythm in *Bryophyllum* to changes in conditions of illumination are complex; its reactions to changes in other external conditions are now under investigation.

ACKNOWLEDGEMENTS

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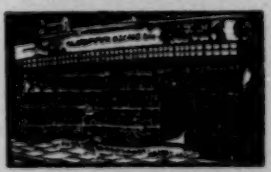

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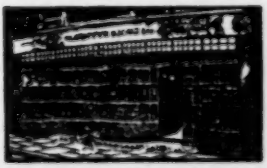

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